BIOACTIVE LIPID SIGNALING IN ENTERIC GLIA

by

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For My Family With All My Love.

Mom and Dad: Thank you for teaching me to dream big.

Christina and Monica: Thank you for tolerating your brother's zany experiments while we were growing up.

Kelly, Gabriel, Olivia and Luke: You keep me grounded. You make me smile and marvel at the beauty of life. Thank you for being my little helpers with the guinea pigs and for making "cell juice" like no one else can. Always remember that being your Dad is infinitely more important to me than my life as a scientist (and I like science a lot!).

Marnie: I am grateful beyond words for both the patience you showed during my long hours in the lab and your faithful encouragement during my endeavor to become a scientist. I couldn't have done it without you.

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CHAPTER 1

INTRODUCTION: CALCIUM SIGNALING IN ENTERIC GLIA

[Note: This chapter has been slightly modified from a manuscript published in the 2001 edition of Recent Research Developments in Neurochemistry, Research Signpost, Inc.]

Abstract

The enteric nervous system serves to regulate the gastrointestinal tract. Within this intricate network, enteric neurons have been previously well characterized whereas enteric glia have not. In recent years, our understanding of enteric glia has increased substantially. Rather than simply playing a passive role in the gut, it now appears that glia act as active, integral components in the enteric nervous system. Like glia of the central nervous system, enteric glia respond to a variety of neuroligands with calcium signaling. Two such agonists are adenosine triphosphate (ATP) and endothelin (ET). In addition to mobilizing intracellular calcium stores through receptor-mediated signal transduction cascades, ATP and ET both also activate capacitative calcium entry. This

review summarizes the current knowledge of signaling events in enteric glia while giving special emphasis towards the effects of ATP and ET upon calcium transients.

Introduction

The enteric nervous system (ENS) is the largest division of the peripheral nervous system (PNS) and is derived embryologically from the neural crest like all other PNS components (1). The ENS, considered a separate class of the autonomic nervous system distinct from sympathetic and parasympathetic branches (2), participates in virtually all aspects of gastrointestinal (GI) function, including blood flow, secretion, absorption, and motility (for reviews see: 3-12). A cross-sectional diagram of the GI tract [Figure 1.1] shows that the ENS is composed of two intrinsic networks, the submucosal plexus (Meissner's plexus) and the myenteric plexus (Auerbach's plexus) [Figure 1.2], each containing interconnected enteric ganglia. The innermost submucosal plexus is situated beneath the intestinal mucosal layer where it regulates epithelial absorption and secretion. The myenteric plexus, embedded between the circular and longitudinal smooth muscle layers, modulates intestinal motility.

Two major cell types comprise the ENS: neurons and glia [Figure 1.3]. Within the ENS, neurons have historically received considerably more investigative attention than glia. Enteric neurons are as numerous as their counterparts in the spinal cord and neural networks have been extensively mapped throughout the GI tract. Classical electrophysiological evaluation of canine intestinal function led to the discovery that the ENS has the ability to locally mediate reflexes, such as the control of peristalsis, without input from the CNS (13). Given its autonomous function, the ENS is also known as, "The Little Brain in the Gut". Although the role of neurons in ENS events is well

established, understanding of enteric glia, while evolving, is much less developed. This review serves to discuss current knowledge of signaling events in these cells.

Enteric Glia -- Active Participants in ENS Signaling

Over two decades ago it became apparent that the enteric nervous system contained a non-neuronal cell type which was quite different from Schwann cells of the PNS and yet similar to astrocytes of the CNS. From a population standpoint, glial cells far outnumber neurons in the GI tract. Morphologically, they resemble CNS astrocytes as they have irregular shapes and do not synthesize a basal lamina (14). At the molecular level, enteric glia stain positively for both the neural crest marker S-100 and, in a fashion similar to astrocytes, glial fibrillary acidic protein (GFAP) (15-17) [Figure 1.4]. As no clear function could initially be linked to these cells, early investigators presumed that glia (from the Greek word meaning "glue") simply performed a supportive role in the gut—as they were once believed to behave in the brain and spinal cord. Indeed, thre have been no published reports of electrophysiological studies of enteric glia to date. Nonetheless, the traditional notion that glia merely serve as passive or nutritive elements within the CNS and ENS is currently being challenged.

Recent work in our laboratory has demonstrated that enteric glia, like CNS astrocytes, are functionally responsive to a variety of neuroligands (15,18-20). The importance of glial cells in the ENS is underscored by the finding that their ablation in transgenic mice precipitates the onset of fatal fulminant jejuno-ileitis (21). Other investigations have suggested that, through aberrations in endogenous ENS receptors

(*e.g.*, endothelin receptor B—ET-B and *RET* proto-oncogene) (22-29) or their designated ligands (*e.g.*, endothelin-3—ET-3 and glial derived neurotrophic factor—GDNF, respectively) (30-32), enteric glia may contribute in the pathogenesis of Hirschsprung's Disease—a congenital condition characterized by varying degrees of ENS maldevelopment and consequent intestinal dysfunction (for reviews see: 33-36). Collectively, these observations suggest that enteric glia may be active participants in ENS signaling and information processing, rather than simply supporting neurons within the continuously moving bowel wall.

Even though enteric glia appear to have an essential role in the ENS, there is a paucity of literature characterizing the molecular mechanisms of signaling in these cells. To this end, we have sought to understand their role in information transfer in the GI tract by focusing upon an important component of signal transduction cascades: calcium signaling. Our studies reveal that a number of compounds cause calcium signaling in enteric glia (15,18) [Table 1.1]. Members of this list include purines, peptides, and non-purinergic/non-peptidergic compounds. This research review will highlight representatives of two different classes of agonists with potent signaling effects in enteric glia: ATP (a purine) and endothelin (a peptide).

Calcium Signaling

The calcium ion (Ca^{2+}) is a critical second messenger that serves to transduce extracellular signals into numerous intracellular events in a variety of cell types. These functions range from short-term responses such as secretion, contraction, intercellular signaling, and motility to longer-term regulation of gene expression, growth, and

apoptosis (for reviews see: 37-50). Glial intracellular Ca^{2+} levels are subject to intricate homeostatic mechanisms which achieve intracellular calcium concentrations ($[Ca^{2+}]_i$) that are roughly 40,000-fold lower than that of the extracellular environment. Under control conditions, basal $[Ca^{2+}]_i$ levels in enteric glia approximate 50 nM vs. 2 mM for extracellular calcium concentrations $[Ca^{2+}]_e$. This steep electrochemical gradient favors Ca^{2+} entry. The Ca^{2+} gradient is attributable to both the sequestration of Ca^{2+} into intracellular stores and mechanisms which export Ca^{2+} from within cells; these measures are accomplished by Ca^{2+}/ATPase pumps in many cells, including CNS glia. Glia are responsive to multiple stimuli which, in turn, initiate numerous downstream effects. Within the ENS a frequent consequence of glial cell stimulation is the generation of temporal and spatial elevations in their cytosolic Ca^{2+} levels—a response known as "calcium signaling".

Calcium signaling is achieved by a variety of mechanisms and culminates in either the influx of extracellular Ca^{2+} ($Ca^{2+}{}_e$) or the mobilization of internal Ca^{2+} ($Ca^{2+}{}_i$) pools. Capacitative calcium entry (CCE) represents yet another aspect of Ca^{2+} signaling which incorporates both events. These concepts are discussed in greater detail in the following sections. Each modality plays an integral role in Ca^{2+} signaling, rendering enteric glia capable of elevating $[Ca^{2+}]_i$ levels into the micromolar range upon stimulation.

Extracellular Calcium Influx

There are two contexts in which $Ca^{2+}{}_e$ plays a role in Ca^{2+} signaling. The first circumstance corresponds to a primary, acute phase of Ca^{2+} signals generated following

cellular stimulation while the latter concerns a secondary, sustained response (i.e., CCE) to the initial release of Ca^{2+}_i stores. Voltage-operated Ca^{2+} channels (VOCCs), ligand-operated Ca^{2+} channels (LOCCs), and mechanosensitive Ca^{2+} channels (MSCCs) contribute to the initiation of cytoplasmic Ca^{2+} transients which utilize Ca^{2+}_e, whereas store-operated Ca^{2+} channels (SOCCs) play a role in CCE.

VOCCs were originally considered to be components of "excitable cells" (*e.g.*, neurons and skeletal or cardiac myocytes—cells which generate action potentials). However, experimental evidence now suggests that CNS glia possess VOCCs. Microfluorometric and electrophysiological approaches have revealed voltage-dependent Ca^{2+} currents in many types of cultured glia, including astrocytes (51-59). Various glial cells also appear to possess ligand-operated Ca^{2+} channels. Best studied of the LOCCs is the AMPA/KA family of ionotropic glutamate receptors (60-65). NMDA receptors in glia are less well understood but remain an active topic of investigation (66,67). Receiving growing attention in glial cells are a unique group of Ca^{2+} channels which are activated upon mechanical stimulation. The current knowledge of MSCCs is also limited, but investigation has been prompted by the observation that focal mechanical stimulation induces Ca^{2+} transients in individual glia and even causes intercellular signaling resulting in the propagation of Ca^{2+} waves in cultured cells (68).

To date, functional VOCCs, LOCCs and MSCCs have not been firmly established in the enteric nervous system. For this reason, our laboratory is interested in their potential involvement in enteric glia, which have many signaling mechanisms common to other glial cells.

Intracellular Calcium Release

The mechanisms leading to the release of Ca^{2+}_i stores have been intensively studied in many cells, including glia. The general signal transduction cascade responsible for Ca^{2+}_i mobilization involves the initial activation of a metabotropic plasma membrane (PM) receptor (typically a G protein-coupled receptor but not infrequently a tyrosine kinase-linked receptor) and subsequent induction of phospholipase C (PLC), which generates the lipid-derived second messengers diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). While DAG activates protein kinase C (PKC), IP_3 causes Ca^{2+}_i release through its receptors which reside on the surface of the endoplasmic reticulum (ER) (for reviews see: 69-71). The PLC-IP_3 machinery appears intact and integral to calcium signaling in enteric glia since pharmacological inhibition of either PLC or the IP_3 receptor selectively blocks Ca^{2+}_i transients in these cells (15,18). Furthermore, the presence of IP_3 receptors in enteric glia has been confirmed by immunofluorescent confocal microscopy (18) [Figure 1.5]. Recently, other intracellular organelles (e.g., mitochondria and the nucleus) have been purported to serve as participants in calcium signaling, but their role in enteric glia remains undefined.

Capacitative Calcium Entry

Capacitative Calcium Entry (CCE) refers to the process of Ca^{2+}_e influx as a direct consequence of the depletion of Ca^{2+}_i stores (72). In the last several years, considerable insight has been gained into understanding this phenomenon (for reviews see: 73-77).

The two principal questions surrounding CCE (also known as "store-operated calcium entry") are: 1) Which calcium channels are involved? and 2) What is the activating signal? Boulay and colleagues showed in the past year that a mammalian homologue of *Drosophila* transient receptor potential channel proteins (*i.e.*, human TRP3) may serve as the SOCC which permits Ca^{2+} entry in CCE (78). Two major competing theories regarding the mechanism of CCE activation have been proposed; they are based upon either the involvement of a diffusible messenger (generically named "calcium influx factor" or CIF) (79,80) or the mechanical linkage of IP_3 receptors to SOCCs through ER-PM interactions (73,81). Most current evidence favors the second scenario, though the mechanism has not been completely elucidated and both remain viable possibilities. In the same investigation that established TRP3 as a SOCC candidate, Boulay *et al.* also provided compelling evidence that TRP channels are activated by a physical coupling with IP_3 receptors. This finding is consistent with the "secretion-like coupling model"—a concept proposed by Patterson *et al.* after discovering that ER-PM interactions are regulated by cytoskeletal modifications in such a manner that CCE, and not IP_3-mediated store release, is selectively affected by F-actin redistribution (83).

CCE has no established physiological function yet is believed to be an integral means of encoding biological cues by transforming acute Ca^{2+} signals into sustained transients.

Recovery Following Calcium Signaling

An important corollary to Ca^{2+}_e influx and Ca^{2+}_i mobilization is the vital role of plasma membrane-bound Ca^{2+} channels which facilitate homeostasis by way of Ca^{2+}_i

extrusion. The temporal nature of Ca^{2+} signals appears to be pertinent in the "coding" of messages and their ultimate termination seems obligatory for preventing initiation of apoptosis signals, which occur following sustained elevations in $[Ca^{2+}]_i$. Ca^{2+} extrusion is chiefly accomplished by energy-dependent plasma membrane Ca^{2+}-ATPases, which terminate calcium signals by re-establishing and maintaining low basal levels of $[Ca^{2+}]_i$. Similar mechanisms exist at the level of the ER for $Ca^{2+}{}_i$ resequestration. Experimental evidence for both recovery routes in enteric glia will be presented in this review in conjunction with the data depicting pre-recovery calcium signaling.

Experimental Methods

Myenteric Plexus Isolation

Since our research focuses upon the enteric nervous system, we have utilized a model of dispersed, mixed primary cell cultures of enteric neurons and glial cells (15). In brief, our technique involves the initial removal of taenia coli from the caecum of neonatal guinea pigs. The taenia coli are then subjected to a collagenase incubation which facilitates microdissection and subsequent isolation of intact myenteric plexuses from within the separated circular and longitudinal muscle layers. Following enzymatic digestion with trypsin, the plexuses are further mechanically dissociated by triturating with siliconized, flamed Pasteur pipets to produce a cell suspension. The cells are cultured on collagen-coated coverslips in complete medium containing 5% New Serum I at 37^oC in 5% CO_2 and used for experiments between days three to ten.

Intracellular Calcium Measurements

After loading with the fluorescent calcium indicator fura 2-AM, cultured cells are examined using a Zeiss Axiovert inverted microscope and an Attofluor imaging system to determine single-cell intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ is calculated from the ratios of the fluoroscence intensities of fura-2 at 334 and 380 nm, monitored by an intensified charge-couple device camera, and subsequently digitized. Calibration of the system is performed with the two-point standardized equation, using fura-2-free acid:

$$[Ca^{2+}]_i = K_d\,[(R-R_{low})/(R_{high} - R)]b$$

where K_d is the dissociation constant of the Ca^{2+}-fura 2 complex (225), R is F_{334}/F_{380}, i.e., the fluorescence at 334 nm excitation divided by the fluorescence at 380 nm excitation, R_{low} is the ratio at zero Ca^{2+} (1 mM EGTA), R_{high} is the ratio at high Ca^{2+} (1 mM $CaCl_2$), and b is F_{380} (zero Ca^{2+})/F_{380} (saturating Ca^{2+}). A ratio pair is taken at every 1.5-3.0 s.

Purinergic Signaling in Enteric Glia—a Role for ATP

Adenosine triphosphate (ATP) has long been appreciated as an important molecule in biological systems. In addition to actions as an intracellular substrate for a vast array of enzymatic reactions, ATP also transduces signals through a family of cell-surface purinergic receptors which were first identified in 1978 (83). While the classification scheme has been modified through the years, the two main classes which Burnstock initially proposed remain to this day; they are: P1 (adenosine) and P2

(ATP/ADP). A subclass of the P2 receptors (P2Y) are G protein-coupled—either partially through pertussis toxin (PTX)-sensitive G proteins (84) or exclusively through toxin-insensitive G proteins (85). Of these, the P2Y2 (formerly known as P2U) receptor has been cloned and localized to a number of normal and transformed cells including the C6 glioma cell line which has been extensively studied as a model of glial cells (84-87).

Our studies have shown that enteric glia undergo robust Ca^{2+} signaling following exposure to purinergic compounds (15) [Figure 1.6]. Given the agonist profile ATP = UTP > ADP > β,γ-methyl-eneadenosine 5'-triphosphate >> 2-methylthioadenosine 5'-triphosphate = AMP = adenosine, we concluded that enteric glia possess a P2Y2 receptor and that the ATP-induced signal was due to stimulation of this receptor.

The Ca^{2+} signaling induced by ATP was dose-dependent over a range of 0.5 to 1000 μM [Figure 1.7]. Additionally, this response was attenuated with repetitive exposure, suggesting that it was a receptor-mediated event. Further evidence in support of this being a receptor-mediated event include the findings that Ca^{2+} responses were decreased 92% by U-73122, an inhibitor of PLC, and 93% by the phorbol ester phorbol 12-myristate 13-acetate (PMA), an activator of PKC. Additionally, depletion of IP_3-sensitive Ca^{2+} stores by the ER Ca^{2+}-ATPase pump inhibitor thapsigargin abolished glial responses to ATP. Pertussis toxin (100 ng/ml, 24 h) did not alter ATP-induced Ca^{2+} signals, indicating that a G protein of the G_i subtype is not involved in this response.

These data imply that glial responses to ATP are mediated by a PTX-insensitive P2Y2 receptor coupled to activation of phospholipase C and release of $Ca^{2+}{}_i$ stores. Importantly, this investigation also provided the first evidence that enteric glia were

functionally responsive to a variety of neurotransmitters, in addition to ATP, including serotonin, bradykinin, histamine.

Peptidergic Signaling in Enteric Glia—a Role for Endothelin

The endothelins are a family of 21-amino acid isopeptides (ET-1, ET-2, ET-3) that were initially linked to vasoreactivity modulation. Two endothelin receptor subtypes (ET_A and ET_B) have been cloned; these receptors have been shown to couple either to PTX-sensitive or PTX-insensitive regulatory G proteins (89). Although endothelins were initially associated with the regulation of cardiovascular function, the identification of endothelin receptors in a variety of other tissues suggests a more heterogeneous array of actions may exist (89). Within the GI tract, endothelins have been reported to influence acetycholine-induced intestinal contractility, colonic secretion, and development of the ENS (90-92,30). Targeted disruption of either the ET_B receptor or its ligand ET-3 results in aganglionic megacolon in animals (23,24,30); corresponding genetic mutations are also found patients with Hirschsprung's disease (22,31). These findings suggest a role for endothelin in the regulation of gastrointestinal function; the involved mechanisms are not completely known.

We have recently reported that each endothelin isoform (ET-1, ET-2, and ET-3) evoked dose-dependent and equipotent increases in $[Ca^{2+}]_i$ (18) [Figures 1.8, 1.9]. Responses to endothelin were inhibited by BQ-788, an ET_B antagonist. Sustained elevation in $[Ca^{2+}]_i$ was abolished by removal of Ca^{2+} from the buffer, inhibited 85% by Ni^{2+}, and diminished by preincubation of glia with PMA by 87%. U-73122 pretreatment abolished the $[Ca^{2+}]_i$ response to ET-3 exposure as did prior exposure of glial cells to

thapsigargin Following introduction of the IP_3 receptor antagonist heparin by radiofrequency electroporation, responses to ET-3 were attenuated by 63% in enteric glia [Figure 1.10]. Preincubation of enteric glia with 100 nM PTX for 24 h failed to inhibit $[Ca^{2+}]_i$ changes in response to 100 nM ET-3.

These data suggest that enteric glia possess the capability of responding to ET with Ca^{2+} signaling via a mechanism involving intracellular and extracellular Ca^{2+}. The events are initiated by a PTX-insensitive ET_B receptor which causes $Ca^{2+}{}_i$ release through PLC and subsequent activation of the IP_3 receptor and also facilitates $Ca^{2+}{}_e$ influx via Ni^{2+}-sensitive PM Ca^{2+} channels.

Capacitative Calcium Entry in Enteric Glia—Effects of ATP and Endothelin

Our investigations have also revealed that in addition to causing Ca^{2+} signaling through $Ca^{2+}{}_i$ release, ATP and ET [Figure 1.11*a*] similarly activate CCE in enteric glia (19,20). When ET was used as an agonist, these responses were blocked by Ni^{2+} (89%) and La^{3+} (78%)—both inorganic, nonspecific inhibitors of Ca^{2+} channels [Figures 1.11*b*-1.11*d*]. Similar results were obtained using ATP. In contrast, neither diltiazem nor ω-conotoxin had any inhibitory effect, suggesting that L-type and N-type Ca^{2+} channels, respectively, were not involved in these responses. Chelerythrine, a specific antagonist of PKC, and the nitric oxide synthase inhibitor N^G-nitro-L-arginine also diminished CCE in response to either agonist.

These data indicate that ATP and ET cause CCE in enteric glial cells via non-L and non-N type Ca^{2+} channels through a process regulated by PKC and nitric oxide.

Conclusions

In summary, enteric glia appear to be more than mere passive elements within the enteric nervous system. Rather, our data illustrate that they are responsive to an array of signaling molecules. ATP and endothelin are two such agonists which cause calcium signaling via cascades incorporating PTX-insensitive plasma membrane receptors (P2Y2 and ET_B, respectively) and PLC activation [Figure 1.12]. In addition to mobilizing intracellular calcium stores, ATP and ET also initiate the subsequent influx of extracellular calcium—capacitative calcium entry. While the physiological role of these signal transduction cascades remains unclear, given the observations made thus far by our lab and others it is tempting to speculate that enteric glia play an active role in information transfer within the GI tract.

Figures

Figure 1.1. Plexuses of the enteric nervous system. Schematic drawing of the wall of the intestine showing the layers which must be removed by microdissection in order to expose the submucosal or myenteric plexuses.

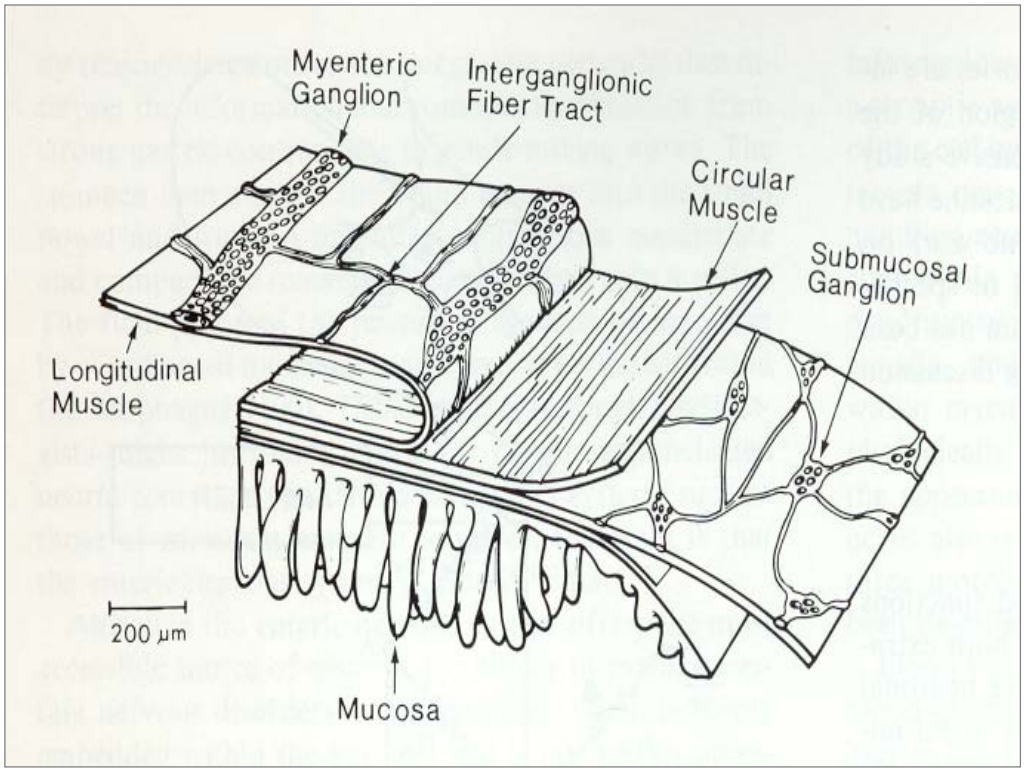
Myenteric
Ganglion
Interganglionic
Fiber Tract
Circular
Muscle
Submucosal
Ganglion
Longitudinal
Muscle
200 μm
Mucosa

FIGURE 1.1

Figure 1.2. Myenteric plexus isolated from neonatal guinea pig taenia coli. Plexus shown was dissected following overnight incubation in collagenase at 4^{o}C and subsequent activation at 37^{o}C for 45 minutes. See accompanying text and Chapter 2 Methods for details.

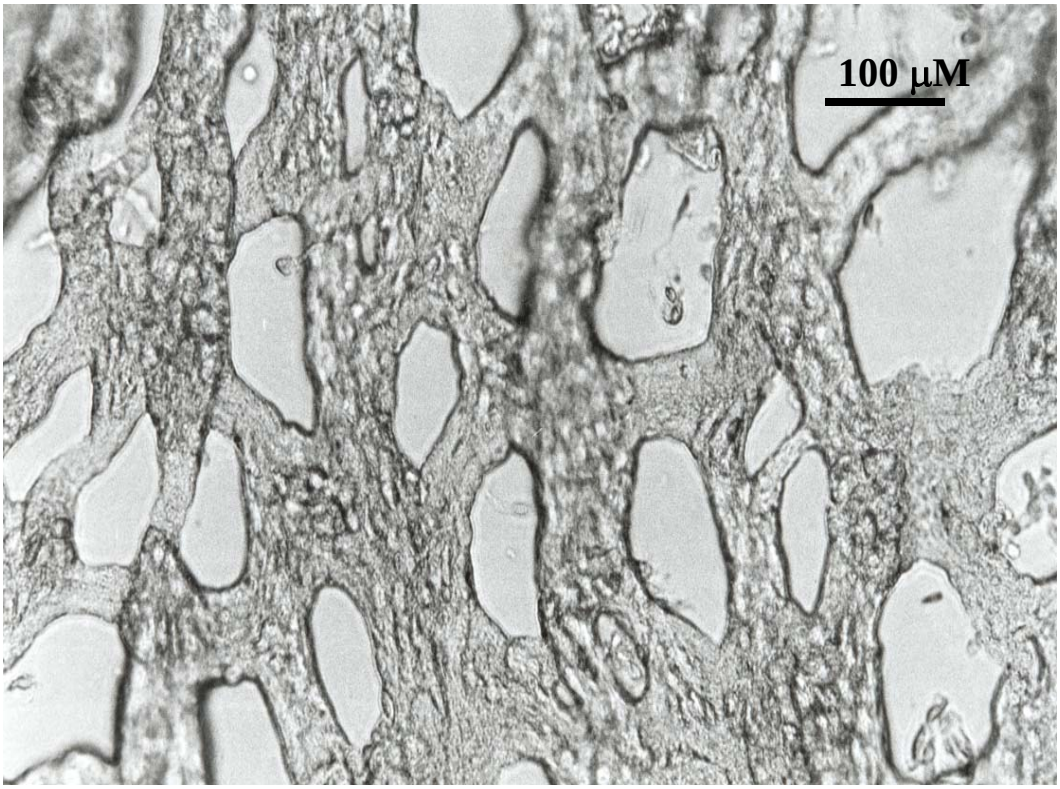

FIGURE 1.2

Figure 1.3. Dispersed primary culture of neonatal guinea pig myenteric plexus depicting enteric neurons (N) and glia (G). Cells shown at day 5 in culture. Note development of intercellular neurite connections.

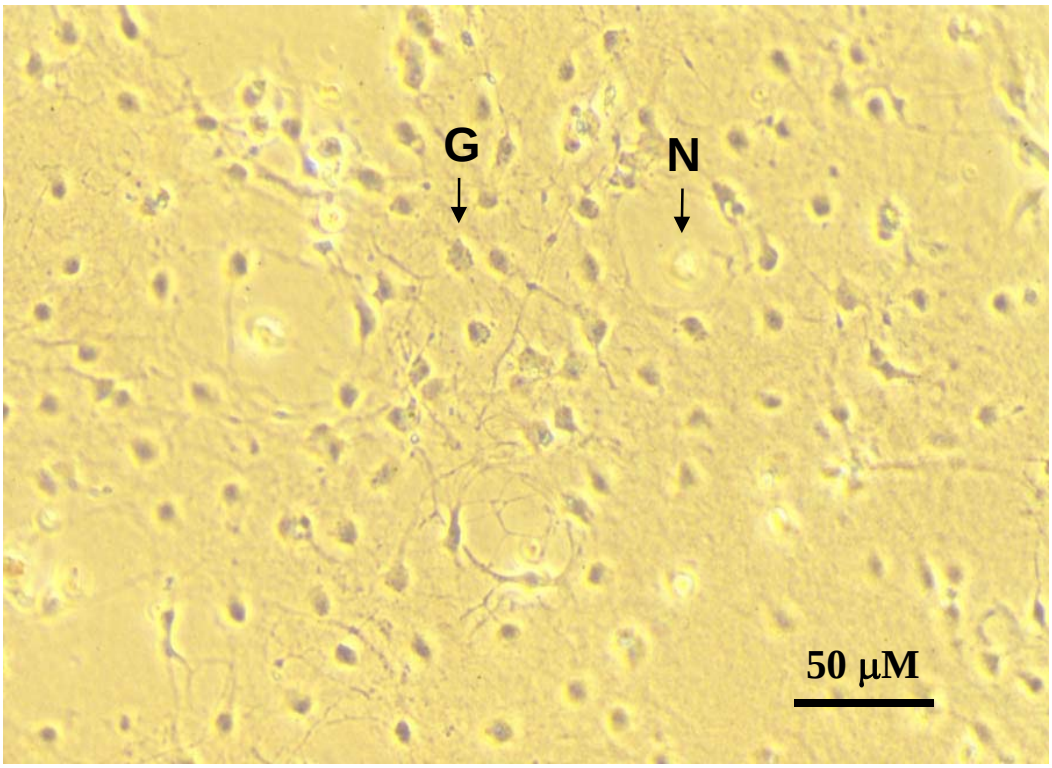

FIGURE 1.3

Figure 1.4. Immunofluorescence staining of GFAP & S-100 in enteric glia. Neonatal guinea pig myenteric plexus cultures from days 2-4 in vitro were incubated with anti-GFAP (primary) and goat anti-mouse fluorescein isothiocyanate fragment (secondary) or anti-S-100 (primary) and streptavidin-Texas red (secondary). Glia stained positively for GFAP (A) and S-100 (B). (X250). Myenteric neurons were unstained.

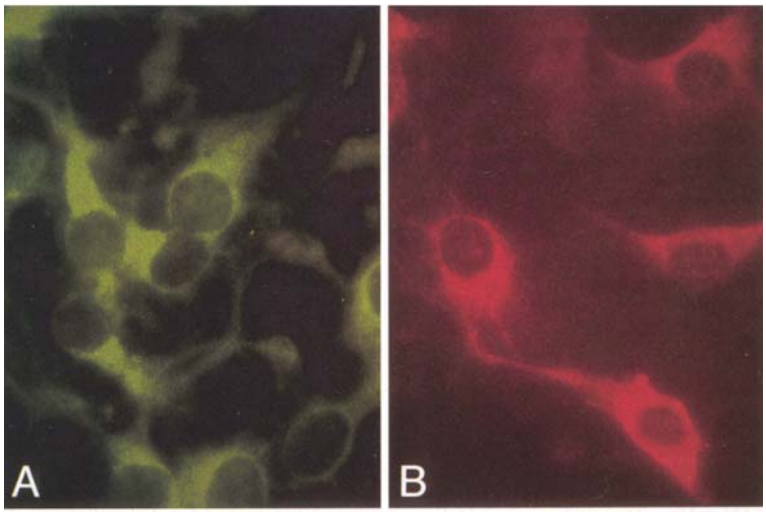

FIGURE 1.4

Table 1.1. Agonist-induced Ca^{2+} signaling in enteric glia.

Compound	Responding Cells (Dose)	Reference
ATP	100% (100 μM)	15
UTP	100% (100 μM)	15
ADP	15% (100 μM)	15
βγ-MeATP	7% (100 μM)	15
Serotonin	4% (100 μM)	15
Bradykinin	11% (10 μM)	15
Histamine	31% (100 μM)	15
Endothelin-1	100% (1 nM)	18
Endothelin-2	100% (1 nM)	18
Endothelin-3	100% (1 nM)	18

TABLE 1.1

Figure 1.5. Immunofluorescence staining of intracellular inositol 1,4,5-trisphosphate (IP_3) receptors in enteric glia. Neonatal guinea pig myenteric plexus cultures at day 3-5 in vitro demonstrate coexistence of IP_3 receptor (fluorescein isothiocyanate, A and C) and S-100 (rhodamine, B and D) in the same cells. No staining was found in cells labeled with control antibodies.

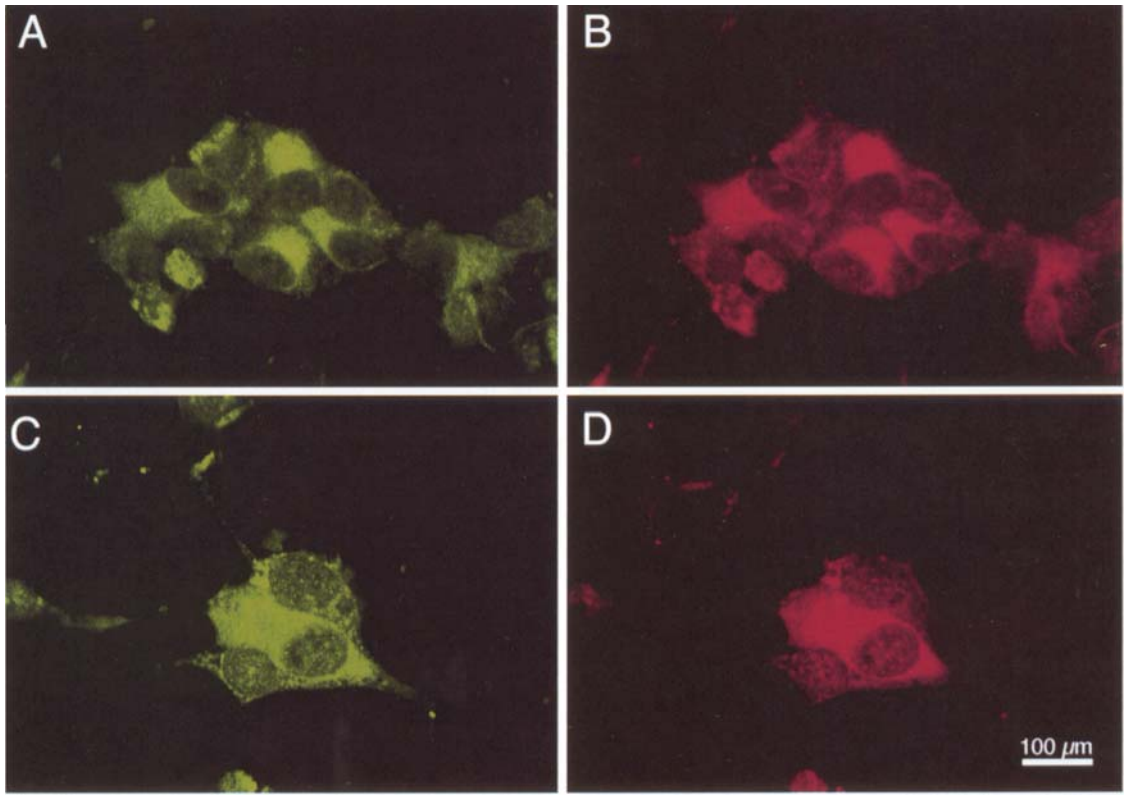

FIGURE 1.5

Figure 1.6. ATP-induced Ca^{2+} signaling. Typical enteric glial response to ATP superfusion (100 μM, 250 s) in a calcium-containing buffer. KCl superfusion (55 mM, 60 s) does not increase $[Ca^{2+}]_i$ in enteric glia; this lack of response in addition to morphology differentiates glial from myenteric neurons during experiments.

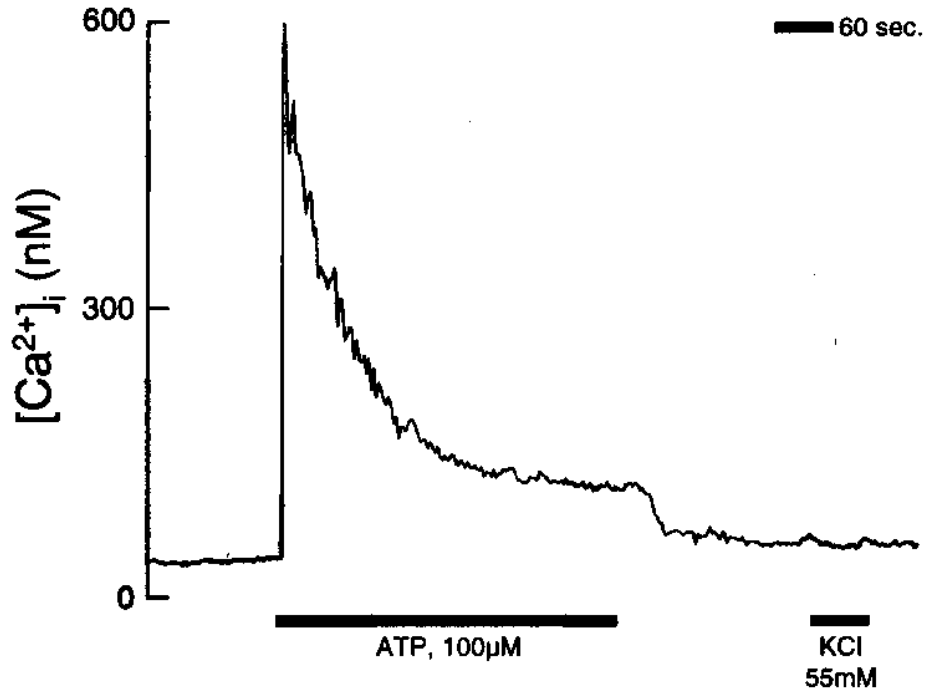

FIGURE 1.6

<u>Figure 1.7.</u> Concentration-dependent increases in peak $[Ca^{2+}]_i$ evoked by ATP in enteric glia. *p<0.05 versus basal value by Student's t test.

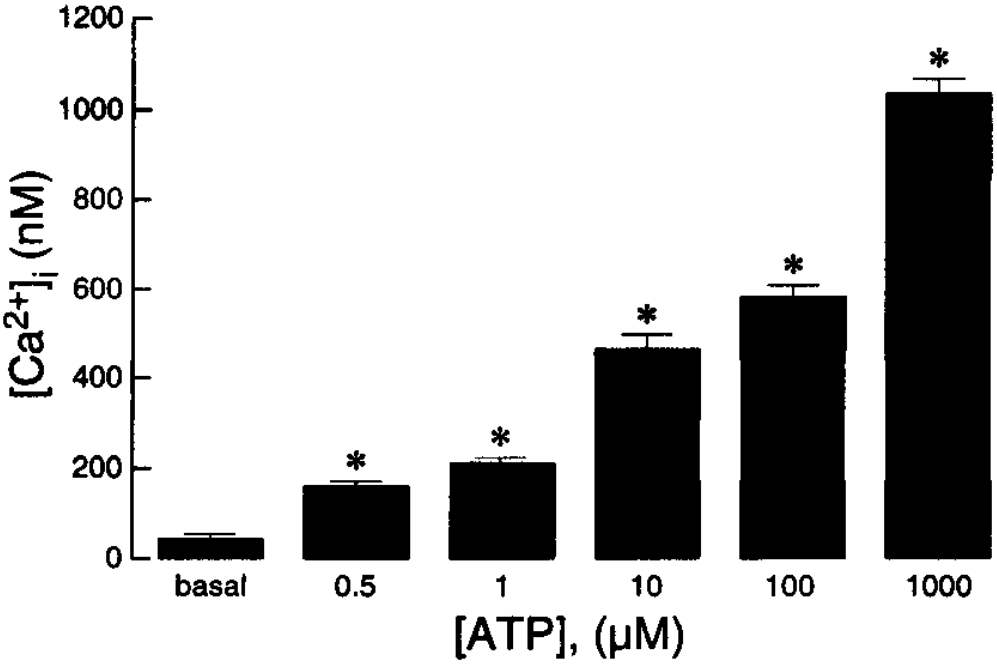

FIGURE 1.7

Figure 1.8. Endothelin (ET)-induced Ca^{2+} signaling. Typical enteric glial responses to superfusion of ET-3 (60 s). A. 0.1 nM ET-3 caused only a transient increase in $[Ca^{2+}]_i$. B. 100 nM ET-3 produced an initial $[Ca^{2+}]_i$ increase followed by a sustained elevation of $[Ca^{2+}]_i$.

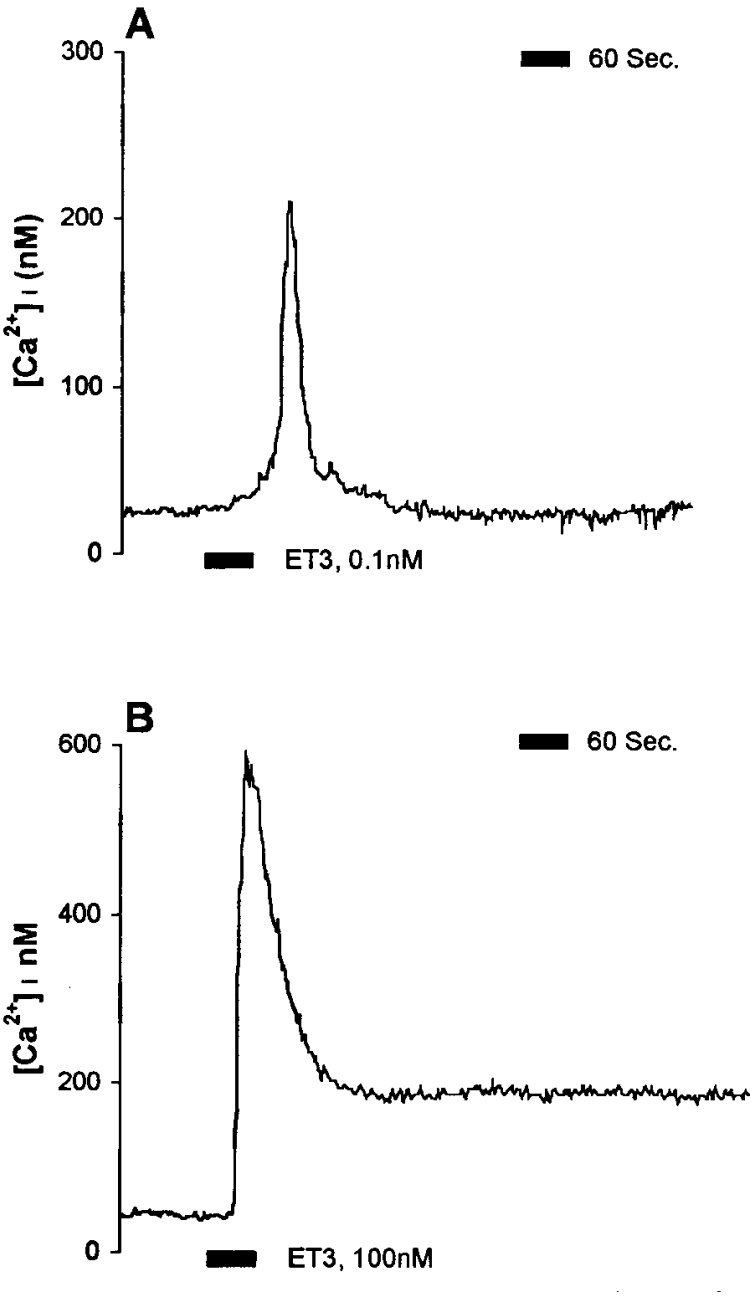
A
300
200
100
0
[Ca2+]i (nM)
60 Sec.
ET3, 0.1nM
B
600
400
200
0
[Ca2+]i nM
60 Sec.
ET3, 100nM

FIGURE 1.8

Figure 1.9. Concentration-dependent Ca^{2+} signaling by ET isotypes 1,2,3. ET-1, ET-2, ET-3 cause dose-dependent increments in peak $[Ca^{2+}]_i$ (A) and in percentage of cells responding (B).

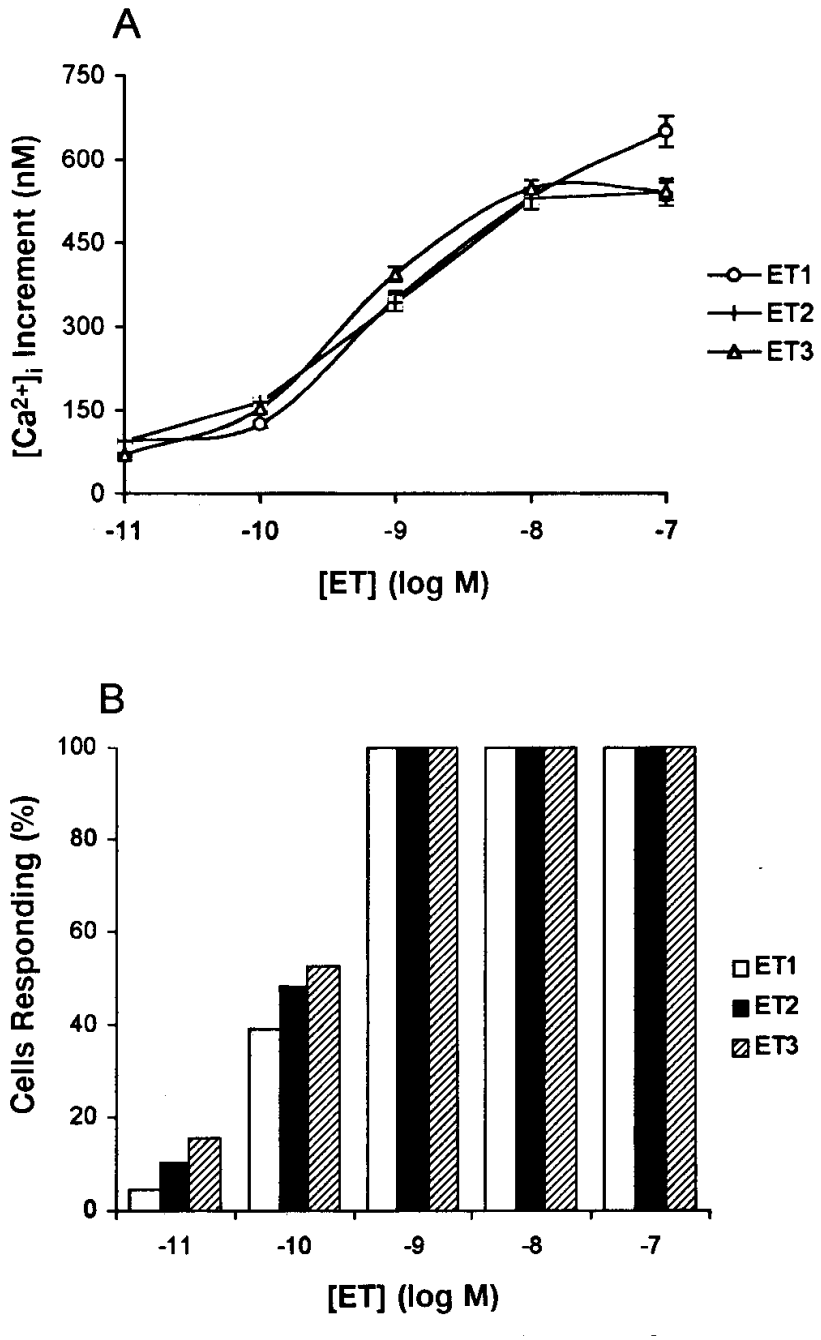
A
750
600
450
300
150
0
[Ca2+]i Increment (nM)
-11
-10
-9
-8
-7
[ET] (log M)
ET1
ET2
ET3
B
100
80
60
40
20
0
Cells Responding (%)
-11
-10
-9
-8
-7
[ET] (log M)
ET1
ET2
ET3

FIGURE 1.9

Figure 1.10. **ET-3 mobilizes IP_3 receptor stores.** Intracellular heparin, an IP_3R antagonist, inhibited ET-3 evoked increases in $[Ca^{2+}]_i$ in enteric glia. A. Fluorescence image of glia taken at 380 nm. Arrows identify position of platinum wire electrodes. B. An image of glia in the same field, viewed under 560 nm wavelength. A loading border, marked by Texas red, identifies electroporated cells (left). C. Pseudocolor image of cells under basal conditions. Both electroporated and non-electroporated cells loaded with fura 2 and maintained similar basal $[Ca^{2+}]_i$ levels. D. On exposure to 100 nM ET-3, glia containing heparin did not demonstrate increases in $[Ca^{2+}]_i$; non-electroporated cells in the same field (right) responded rapidly to ET-3 exposure. Electroporation of chondroitin sulfate, which approximates the size and charge of heparin, had no effect on ET-3 responses.

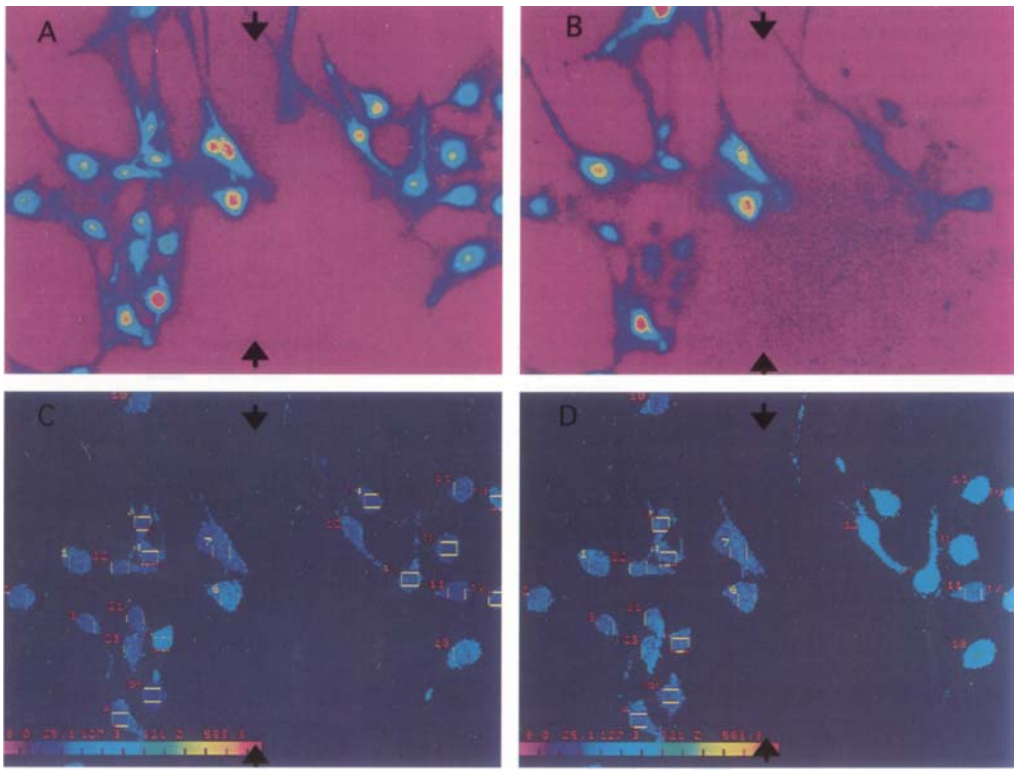

FIGURE 1.10

Figure 1.11. **ET-3 causes capacitative calcium entry (CCE).** A. Depletion of intracellular Ca^{2+} stores by 10 nM ET-3 in Ca^{2+}-free buffer causes subsequent CCE on addition of Ca^{2+} to the perfusion buffer. B. Superfusion with 1 mM Ni^{2+} inhibited the increase of $[Ca^{2+}]_i$ when Ca^{2+} was added to the perfusion buffer; $[Ca^{2+}]_i$ increased when Ni^{2+} was removed. C. Similar to Ni^{2+}, La^{3+} reversibly inhibited CCE. D. Summary of inhibitory effects of Ni^{2+} and La^{3+}. *$p<0.05$ versus control by Student's t test.

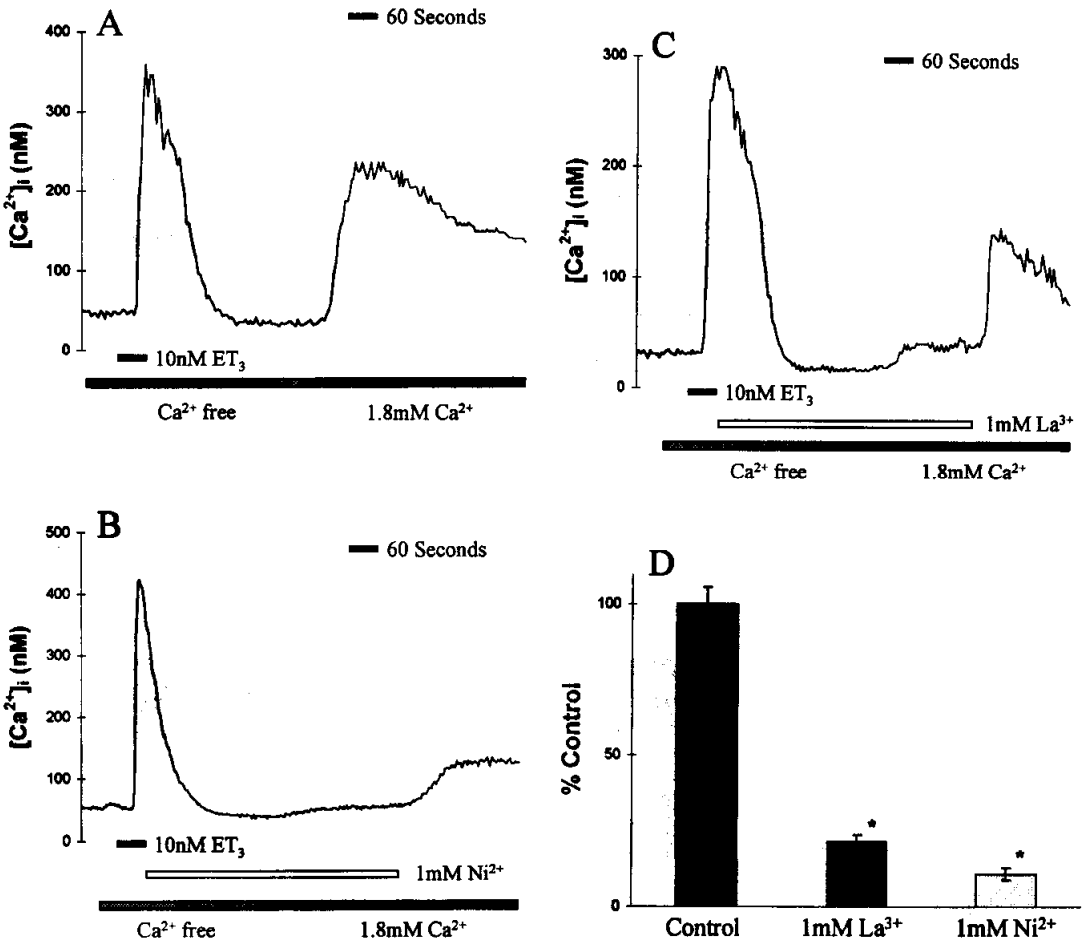
A
60 Seconds
400
300
200
100
0
[Ca2+]i (nM)
10nM ET3
Ca2+ free
1.8mM Ca2+
C
60 Seconds
300
200
100
0
[Ca2+]i (nM)
10nM ET3
1mM La3+
Ca2+ free
1.8mM Ca2+
B
60 Seconds
500
400
300
200
100
0
[Ca2+]i (nM)
10nM ET3
1mM Ni2+
Ca2+ free
1.8mM Ca2+
D
100
50
0
% Control
Control
1mM La3+
1mM Ni2+

FIGURE 1.11

<u>Figure 1.12.</u> Summary model of ATP- and ET-induced Ca^{2+} signaling in enteric glia. CCE=Capacitative Calcium Entry, ER=endoplasmic reticulum, IP_3=1,4,5-inositol trisphosphate, PIP_2=phosphatidyl inositol bisphosphate, PLC=phospholipase C. See text for details.

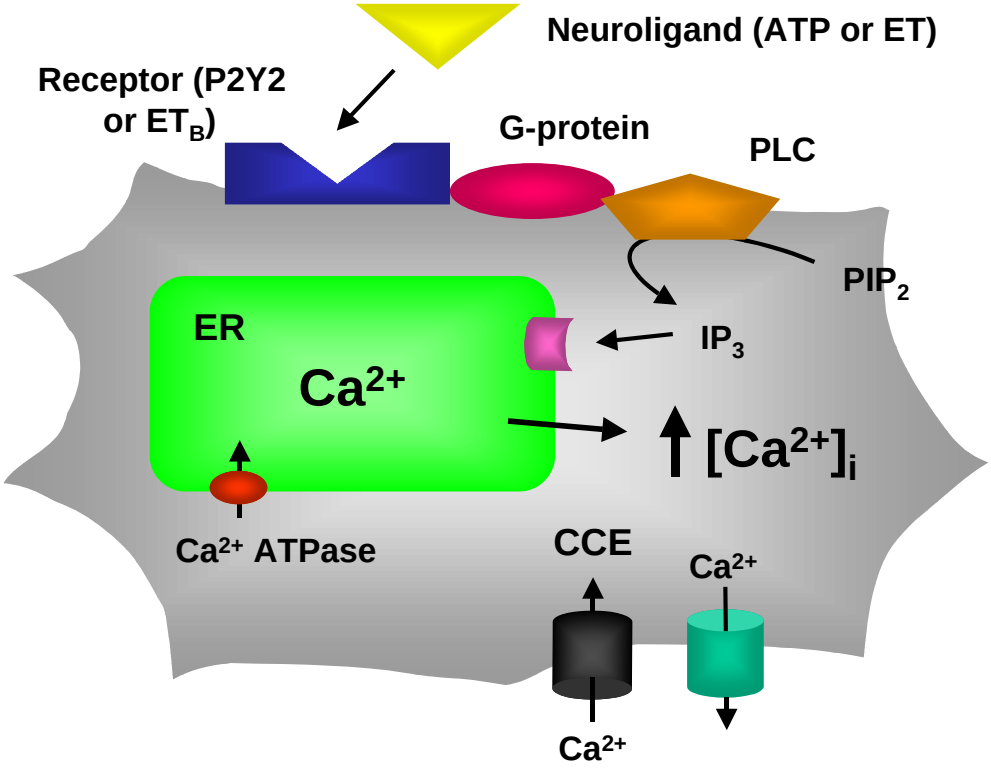
Neuroligand (ATP or ET)
Receptor (P2Y2 or ET_B)
G-protein
PLC
PIP_2
ER
Ca^{2+}
IP_3
$[Ca^{2+}]_i$
Ca^{2+} ATPase
CCE
Ca^{2+}
Ca^{2+}

FIGURE 1.12

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CHAPTER 2

SPHINGOSINE-1-PHOSPHATE AND RELATED BIOACTIVE LIPIDS SELECTIVELY MEDIATE CALCIUM SIGNALING IN GLIAL CELLS OF THE ENTERIC NERVOUS SYSTEM

[Note: This chapter has been slightly modified from a published manuscript: Segura, B.J., Zhang, W., Xiao, L., Turner, D., Cowles, R.A., Logsdon, C., & Mulholland, M.W. 2004. Sphingosine-1-phosphate mediates calcium signaling in guinea pig enteroglial cells. *J. Surg. Research* 116(1): 42-54.]

Abstract

The enteric nervous system (ENS) regulates the gastrointestinal tract. Within this intricate network, enteric glia play a crucial role in maintaining normal bowel integrity, yet their signaling mechanisms are only beginning to be understood. We studied the actions of a novel lipid compound in the ENS with the long-term goal of understanding neurotransmission within the gut. Here we report that enteric glia, and not enteric neurons, respond to a unique class of molecules: bioactive lipids. Of these, the sphingomyelin metabolite sphingosine-1-phosphate (S1P) causes dose-dependent calcium

(Ca^{2+}) signaling utilizing extracellular and intracellular Ca^{2+}. Although pertussis toxin-insensitive, this signal transduction cascade appears to involve an extracellular receptor since repetitive exposure yields diminished responsiveness and inhibition of either phospholipase C or the inositol 1,4,5-trisphosphate receptor abolishes S1P effects. Further supporting a receptor-mediated event, RT-PCR analysis demonstrates the presence of several S1P-coupled endothelial differentiation gene (EDG) receptor mRNAs (EDG-1, EDG-3, and EDG-5) within the ENS. Immunocytochemical analysis demonstrated strong expression of both EDG-1 and EDG-3 and weak expression of EDG-5 in enteric glial cells. As other sphingomyelin cycle components (sphingomyelin, sphingomyelinase, and sphingosine) also activate Ca^{2+} signaling in enteric glia, we postulate that these cells are capable of processing sphingolipids to elicit intracellular S1P responses. Additionally, the finding that related lipids lysophosphatidic acid and sphingosylphosphorylcholine—agonists of differing EDG receptors—also induce Ca^{2+} signaling in enteric glia suggests multiple lipid-activated signaling mechanisms exist in these cells. We propose that S1P and other bioactive lipids function as novel signaling molecules within the ENS to facilitate information transfer.

Introduction

The enteric nervous system (ENS) is the largest division of the peripheral nervous system, and modulates virtually all aspects of gastrointestinal (GI) function, including blood flow, secretion, absorption, and motility (1). Within the ENS, glial cells far outnumber neurons and are similar to astrocytes of the central nervous system (CNS) at morphological and molecular levels (2-4). The traditional notion that glia merely serve as passive or nutritive elements within the CNS and ENS is currently being challenged (5). Recent studies have revealed that enteric glia, like CNS astrocytes, are functionally responsive to a variety of neuroligands (6-9). The importance of glial cells in the ENS is further substantiated by the finding that adult transgenic mice develop fulminating and fatal jejuno-ileitis following ablation of enteric glia (10). Additional investigations indicate that, as a consequence of abnormalities in endogenous ENS receptors (11-18) or their designated ligands (12, 19-21), enteric glia may contribute in the pathogenesis of Hirschsprung's disease. These observations collectively suggest that enteric glia play an active role in ENS signaling and information processing to preserve normal GI function, rather than simply supporting neurons within the continuously moving bowel wall.

Sphingosine-1-phosphate (S1P) has received increasing attention as a member of a new class of lipid signaling molecules (22). S1P is generated through sphingomyelin catabolism and has been implicated in an array of cellular events including calcium (Ca^{2+}) signaling (5, 23, 24). S1P is distinctive because it may serve as both an intracellular and an extracellular mediator of Ca^{2+} mobilization (25- 30). The recent identification of a subfamily of G protein-coupled receptors with affinity for S1P has

sparked interest in the role of this lipid as an extracellular messenger (23, 27, 28, 31-34). A role for S1P and other bioactive lipids in the ENS has not been established.

Enteric glia were postulated to be responsive to sphingolipids and the mechanisms by which S1P affects intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were examined in these cells. The current studies demonstrate that in enteric glia: 1) numerous sphingomyelin cycle components and related lipids selectively cause calcium signaling; 2) S1P elicits dose-dependent Ca^{2+} signaling in the percentage of responding cells and in the incremental rise in $[Ca^{2+}]_i$; 3) S1P-induced Ca^{2+} responses desensitize with repetitive exposure; 4) S1P effects are not inhibited by pertussis toxin exposure; and 5) S1P mobilizes thapsigargin- and inositol-1,4,5-trisphosphate-sensitive intracellular Ca^{2+} stores through activation of phospholipase C. Myenteric plexus cultures were demonstrated to express EDG-1, EDG-3 and EDG-5 receptor mRNA, and to be positive immunocytochemically for EDG-1, EDG-3 and, to a lesser degree, EDG-5.

Experimental Methods

Materials and Reagents

Sphingosine-1-phosphate (S1P), D-erythro-sphingosine (D-SPH), L-erythro-sphingosine (L-SPH), lysophosphatidic acid (LPA), sphingosylphosphorylcholine (SPC), sphingomyelin (SM), sphingomyelinase (SMase), adenosine triphosphate (ATP), collagenase type V, thapsigargin, U73122, U73343, trypsin-EDTA, soybean trypsin inhibitor (type I-S), penicillin-streptomycin solution, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and saponin were from Sigma Chemical (St. Louis, MO).

C2-ceramide (C2-CER) was from Cayman Chemical (Ann Arbor, MI). Pertussis toxin (PTX) was from Calbiochem (San Diego, CA). Hanks' balanced salt solution (HBSS), medium 199, and L-glutamine were from GIBCO BRL (Grand Island, NY). Rat tail collagen was from Boehringer Mannheim (Mannheim, Germany). New Serum I (NSI) was from Collaborative Research (Bedford, MA). 2-aminoethoxydiphenyl borate (2-APB) was from Tocris (Ballwin, MO). Fura-2-AM, fluorescein conjugated streptavidin (S-869), DAPI cytological nuclear conterstain kit (C-7590) and prolong antifade kit (p-7481) were from Molecular Probes, Inc. (Eugene, OR). TRIzol reagent, DNAse I, SuperScript II RNase H^- Reverse Transcriptase (RT), and RT buffer were from Life Technologies, Inc. (Grand Island, NY). Deoxynucleotides (dNTP mix) and oligo d(T) primers were from Roche Molecular Biochemicals (Indianapolis, IN). RNase inhibitor was from Promega (Madison, WI). Advantage cDNA polymerase mix and PCR reaction buffer were from Clontech Laboratories, Inc. (Palo Alto, CA). One-day-old male Duncan-Hartley guinea pigs were obtained from Simonsen Labs (Gilroy, CA).

EDG1 C-terminus rabbit polyclonal antibody (Ab) (X1093P), EDG1 blocking peptide (X1224B), EDG3 N-terminus mouse monoclonal Ab (C175M) and EDG5 C-terminus mouse monoclonal Ab (C190M) were from Exalpha Biologicals, Inc. (Boston,MA). Normal goat serum (sc-2043), biotinylated goat anti-mouse IgG (sc-2039) and biotinylated goat anti-rabbit IgG (sc-2040) were from Santa Cruz Biotechnology, Inc.(Santa Cruz, CA).

Myenteric Plexus Isolation

Dispersed primary cultures of guinea pig myenteric plexus were prepared on collagen-coated coverslips and used for experiments within 10 days post-plating using a protocol refined in our laboratory (6). One- to three-day-old Duncan Hartley guinea pigs were sacrificed and their taenia coli were removed using sharp dissection with tissue forceps and Castro-Viejo scissors then placed in Hanks Balanced Salt Solution supplemented with 0.1% Type IV collagenase for 16-24 h at 4^{o}C. After a 30-60 minute incubation at 37^{o}C, the muscle layers of the taenia coli were separated from the myenteric plexus with the use of fine tissue forceps and a dissecting microscope. The myenteric plexus was trypsinized for 30 min at 37^{o}C using 0.1% trypsin-EDTA solution, triturated with siliconized flamed Pasteur pipettes of decreasing diameter, and plated on aseptically-prepared collagen-coated 22 mm glass coverslips in a 35 mm culture dish using sterile technique. Cultures were exposed to complete medium 199 plus 5% NSI and 0.01% trypsin inhibitor. Penicillin-streptomycin solution was added for the first 48 h at a 2% concentration. Antimitotic agents were not added. Medium was changed every 24-36 hours. The cultures were incubated at 37^{o}C with 5% CO_2. All animals were used in accordance with established guidelines and protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan Medical Center (approval no. 7340).

Solutions

All experiments were performed in standardized solutions except when noted. Standard control buffer was a modified Krebs-Ringer solution at pH 7.40 containing (in mM) 118 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 10 HEPES, 15 $NaHCO_3$, 11 glucose, 0.9 NaH_2PO_4, and 0.8 $MgSO_4$. In Ca^{2+}-free control buffer, the $CaCl_2$ was omitted and 0.5 mM EGTA was added.

Loading and Cell Preparation for Imaging

For loading purposes, fura 2-AM was added to achieve a final concentration of 2 μM for 45 min to cultured myenteric plexus and incubated at 37^0C in serum-containing growth media described above that was changed no less than 24 hours prior to avoid acute effects of new serum supplementation. Loaded coverslips were washed and then stored in serum-free control buffer and placed in a lucite superfusion chamber (approximate volume 0.5 ml). The superfusion rate of the control buffer and experimental solutions was 1 ml/min at 37^oC.

Ca^{2+} Measurements

A Zeiss Axiovert inverted microscope and an Attofluor imaging system (Rockville, MD) were used to determine single-cell intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was calculated from the ratios of the fluorescence intensities of fura-2 at

334 and 380 nm, monitored by an intensified charge-couple device camera, and subsequently digitized. Calibration of the system was performed with the two-point standardized equation, using fura-2-free acid:

$$[Ca^{2+}]_i = K_d\,[(R\text{-}R_{low})/(R_{high} - R)]b$$

where K_d is the dissociation constant of the Ca^{2+}-fura 2 complex (225), R is F_{334}/F_{380}, i.e., the fluorescence at 334 nm excitation divided by the fluorescence at 380 nm excitation, R_{low} is the ratio at zero Ca^{2+} (1 mM EGTA), R_{high} is the ratio at high Ca^{2+} (1 mM $CaCl_2$), and *b* is F_{380} (zero Ca^{2+})/F_{380} (saturating Ca^{2+}). Frames were not averaged to obtain images. The system was used to obtain whole field images. Boxes were placed on all cells of interest for simultaneous measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). A ratio pair was taken at every 1.5-3.0 s.

RNA Extraction and Reverse Ttranscription (RT)

Total cellular RNA was isolated from 5 day-old myenteric plexus primary cell cultures using TRIzol reagent according to manufacturer's directions. Single strand cDNA synthesis was performed as follows: 20 µl of reverse transcription mixture contained 1 µg of DNase I pre-treated total RNA, 0.5 µg of oligo d(T) primer, 4 µl of 5 x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 40 units of RNase inhibitor, and 200 units of reverse transcriptase (SuperScript II RT). The RT reaction was carried out at 42^{o}C for 50 min followed by heat inactivation at 70^{o}C for 15 min.

Polymerase Chain Reaction (PCR)

50 μL of PCR reaction mixture contained 1 μl of RT products, 1X cDNA PCR reaction buffer, 400 nM of each primer, 200 μM of dNTP mix, and 1X Advantage cDNA polymerase mix. The PCR was carried out using a Perkin-Elmer Thermal Cycler (Norwalk, CT). Samples were denatured initially at 94^{o}C for 1 min and the PCR was performed as follows: 38 cycles of 35 sec at 94^{o}C, 1 min at 62^{o}C and 1 min 40 sec at 72^{o}C for EDG-1; 38 cycles of 35 sec at 94^{o}C, 1 min at 67^{o}C and 1 min 20 sec at 72^{o}C for EDG-3; 38 cycles of 35 sec at 94^{o}C, 2 min 15 sec at 68^{o}C for EDG-5, following by the final extension at 72^{o}C for 7 min.

PCR primers used for guinea pig mRNA detection were deduced from human, mouse and rat published sequences. The nucleotide sequences of sense and antisense primers with the expected product size are follows: EDG-1, TAT GAT ATC ATA GTC CGG CAT TAC A (sense) and GCA TCT CCT TGT TGG TCA GAG TGT (antisense) (892 bp product); EDG-3, CTC AGG GAG GGC AGT ATG TTC GT (sense) and TCC TTG ACC TTC GGA GAG TGG CTG (antisense) (715 bp product); EDG-5, GAA CAC TAC AAT TAC ACC AAG GAG ACG C (sense) and ACG ATG GTG ACC GTC TTG AGC AGG GC (antisense) (667 bp product). For negative controls, PCR reactions were performed for each of the primer pairs in the absence of transcript as others have reported.

Sequencing and Data Analysis

PCR products were electrophoretically analyzed on 1.5% agarose gel containing ethidium bromide. DNA bands were excised from the gel and purified as follows: gel slices were crushed in the tube and sample DNA was extracted twice with buffered phenol and once with phenol:chloroform:isoamyl (25:24:1) followed by ethanol precipitation with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol and washing with 70% ethanol. The purified DNA fragments were directly sequenced by the DNA sequencing core at the University of Michigan using Applied Biosystems DNA sequencers.

Immunochemistry

Cultured guinea pig myenteric plexus cells were used on day 5 or day 6. Cells were fixed in freshly prepared 4% paraformaldehyde/PBS (pH 7.4) for 20 min at 4^{O} C (all steps were performed at 4^{o}C unless otherwise noted. Following rinsing in PBS, the cells were incubated with blocking/permeabilization buffer (10% normal goat serum, 2% BSA and 0.1% saponin in PBS) for 20 min at room temperature. After washing with PBS, the cells were incubated with primary antibody solution overnight at 4^{o}C. Mouse monoclonal antibody was diluted at 1:50 in PBS with 1.5% goat serum. Rabbit polyclonal antibody was diluted at 1:100 in PBS with 1.5% goat serum. As a negative control, rabbit polyclonal antibodies were pre-incubated with specific blocking peptides for 20 min at 37^{O} C; the sample without primary antibody was used for negative control for mouse monoclonal antibody samples. After thoroughly a wash with PBS, the samples

were incubated for 45 min with biotinylated goat anti-mouse or anti-rabbit antibody (1:200 diluted in PBS with 1.5% goat serum), illuminated by reaction with fluorescein conjugated streptavidin, stained with DAPI cytological nuclear conterstain kit and mounted with prolong antifade kit following company instructions. Samples were examined using a Nikon microscope with epifluorescence capabilities and images were recorded using a Spot camera then processed in Adobe photoshop.

Data Presentation and Calculation

Results are expressed as mean ± SEM. Data were analyzed using an unpaired Student's *t*-test. Significance was accepted as $P < 0.05$ (95% confidence interval).

Dissection techniques, tissue preparation, media, and reagent vendors remained constant throughout the study. In this study, *n* equals the number of glial cells examined. At least three coverslips were used for each experimental condition. All experimental conditions were examined on glial cells derived from cell preparations performed on at least two different days.

Results have been calculated only from those responding glia having basal $[Ca^{2+}]_i$ levels < 100 nM, a criteria met by > 95% of glial cells. Cells were considered to be responsive if peak $[Ca^{2+}]_i$ was at least 50 nM higher than the baseline value. Glia with a high $[Ca^{2+}]_i$ before any addition of agonist were considered damaged or leaky and were excluded from the study. Only one microscope field was examined per coverslip. Peak $[Ca^{2+}]_i$ was measured as the highest $[Ca^{2+}]_i$ achieved during agonist exposure. $\Delta[Ca^{2+}]_i$ represents the difference between peak and basal $[Ca^{2+}]_i$.

At the time of the experiments, two criteria were used to determine whether cells of interest were glial cells, as opposed to neurons. *1)* Morphology. Myenteric neurons 2-7 days post-plating are compact and phase-bright, with few or no processes. Enteric glia have a larger, dense nucleus with wide surrounding cytoplasm. *2)* KCl depolarization. At the end of each experiment, the coverslip was superfused with 55 mM KCl. Enteric glia typically do not exhibit increments in $[Ca^{2+}]_i$ on exposure to 55 mM KCl.

Experimental Results

Sphingolipid Analogs Cause Ca^{2+} Signaling in Enteric Glia.

To determine whether cultured guinea pig enteric glial cells are responsive to bioactive lipids, mixed primary cultures of myenteric plexus were exposed to an array of related lipid compounds that are depicted in a summary of the biochemical metabolism of sphingomyelin (SM) [Figure 2.1]. The compiled data of these bioactive lipids and other components of the SM cycle are displayed in Figure 2.2. Perfusion of glial cells with SM (1 μM) for 300 s caused Ca^{2+} mobilization in 5±6% of glial cells (n=119). Exposure of cells to SMase for 300 s (0.1 U/ml) produced increments in $[Ca^{2+}]_i$ in 48±31% of cells (n=109), suggesting that enzymatic generation of sphingolipid signaling molecules can elicit calcium signaling. C2-CER (1 μM and 10 μM), a truncated form of ceramide that retains biological activity yet is less likely than full length ceramide to be further metabolized, did not produce increases in $[Ca^{2+}]_i$ within five minutes of exposure (96 and 144 cells examined, respectively). D-SPH (10 μM) exposure for 300 s caused $[Ca^{2+}]_i$

increments of 108±4 nM with 28±9% of cells responding (150 cells examined). For control of non-specific effects, an inactive enantiomer of sphingosine, L-SPH (10 μM), was also investigated. L-SPH did not induce Ca^{2+} signaling in any of the cells examined (n=184). S1P (1 μM) superfusion produced Ca^{2+} transients [Figure 2.3] in 80±14% of cells ($\Delta[Ca^{2+}]_i$=203±10 nM; n=143) examined over 300 seconds. LPA (1 μM) and SPC (1 μM) also generated Ca^{2+} signals of 141±5 nM and 161±9 nM in 85±6% (n=114) and 66±20% (n=128) of enteric glia, respectively. Enteric neurons were not found to respond with Ca^{2+} signaling to any of the lipids investigated, including S1P [Figure 2.4]. Further suggestive of neuronal-independent signaling by S1P, pre-treatment with the depolarizing agent tetrodotoxin (1 μM) for ten minutes had no effect upon S1P transients.

S1P-mediated Ca^{2+} Signaling is Dose-Dependent and Desensitizes with Repetitive Exposure.

S1P produced dose-dependent increments in percentage of cells responding [Figure 2.5*a*] and in $[Ca^{2+}]_i$ increments [Figure 2.5*b*] over the range of 1 nM to 10 μM. For remaining experiments, 1 μM S1P was chosen as a suitable working dose. Repetitive exposure to S1P produced progressive decrements in peak $[Ca^{2+}]_i$ in enteric glia [Figure 2.6*a*]. An approximately 31% decrease in peak $[Ca^{2+}]_i$ responses was observed between first and second S1P exposures, and another decrement of 16**%** between the second and third exposures [Figure 2.6*b*]. 88±6% of cells responded to S1P and none completely desensitized (n=116). To demonstrate that the effect was not due to depletion of

intracellular Ca^{2+} stores, cells were subsequently perfused with ATP (100 μM) at the end of each experiment and significant increments in $[Ca^{2+}]_i$ were achieved.

S1P Responses are Pertussis Toxin (PTX)-Insensitive.

To investigate the involvement of a PTX-sensitive G protein, enteric glia were preincubated with PTX at 100 ng/ml for 24 h, a concentration and preincubation time found to be inhibitory in other studies (35, 36). $\Delta [Ca^{2+}]_i$ generated by S1P in glia pre-exposed to PTX was 137±7 nM (n=133), relative to the $\Delta [Ca^{2+}]_i$ evoked in control glia of 151±7 nM (n=153). The percentage of cells responding to S1P was also similar in these two groups: 81±3% vs. 82±3% for PTX-treated and non PTX-treated cells, respectively.

S1P Mobilizes Intracellular Ca^{2+} Stores.

To evaluate the role of intracellular Ca^{2+} ($Ca^{2+}{}_i$) stores in S1P-generated calcium signaling, responsiveness of enteric glia to S1P was investigated using Ca^{2+}-free medium supplemented with 1 mM EGTA. Exposure to S1P in a nominally Ca^{2+}-free medium had no effect on percentage of cells responding or on peak $[Ca^{2+}]_i$, suggesting that the initial Ca^{2+} response is generated from $Ca^{2+}{}_i$ stores. The morphology of the observed Ca^{2+} transients, however, were distinctly different [Figure 2.7]. The absence of extracellular Ca^{2+} ($Ca^{2+}{}_e$) abolished the sustained plateau in intracellular $[Ca^{2+}]$, indicating that an influx of $Ca^{2+}{}_e$ occurs following initial $Ca^{2+}{}_i$ mobilization. Thapsigargin, an inhibitor of

the endoplasmic reticulum Ca^{2+}-ATPase, depletes $Ca^{2+}{}_i$ stores without generating IP_3. During superfusion with thapsigargin (1 μM) in a Ca^{2+}-free medium, all cells (n=123) responded with increases in $[Ca^{2+}]_i$, followed by return of $[Ca^{2+}]_i$ to baseline; at this point, $Ca^{2+}{}_i$ stores were presumed to be depleted. Subsequent exposure to S1P produced Ca^{2+} transients in none of the glial cells examined [Figure 2.8].

S1P Activates Phospholipase C (PLC).

The aminosteroid U-73122 has been shown to inhibit PLC-coupled responses in a variety of cells. Pretreatment of myenteric glia with U-73122 (1 μM) for 300 s abolished subsequent S1P-induced Ca^{2+} signaling (5±4% of experimental cells responding vs. 89±5% for control cells) [Figure 2.9; *n*=171 and 145, respectively]. The inactive analogue of U-73122 (U-73343, 1 μM) had no effect on S1P-mediated Ca^{2+} transients [Figure 2.9; *n*=170], suggesting the effect of U-73122 was not due to nonspecific cytoxicity.

S1P-induced $Ca^{2+}{}_i$ Mobilization Involves the Inositol 1,4,5-trisphosphate (IP_3) Receptor (IP_3R).

The role of IP_3-sensitive intracellular Ca^{2+} stores in S1P signaling was investigated using the membrane-permeable IP_3R antagonist 2APB. 2APB has been utilized in a variety of cell types to reversibly modulate IP_3R activity independent of IP_3 production and binding, voltage-gated calcium channels, or ryanodine-sensitive calcium

stores (37, 38). In this investigation, 2APB had no effect on basal cytosolic [Ca^{2+}] at any of the doses evaluated. 2APB caused dose-dependent inhibition of S1P-induced Ca^{2+} signals in enteric glia, nearly abolishing responsiveness at a concentration of 200 μM [Figure 2.10]. Following pretreatment with 2APB (200 μM X 5 min), only 6±6% of glia (n=161) responded to S1P exposure vs. 92±3% for controls (n=156).

EDG-1, EDG-3 and EDG-5 mRNAs are Expressed in the ENS.

To further assess the participation of S1P-responsive G protein-coupled receptors of the endothelial differentiation gene family, RT-PCR analysis was performed using primers generated from known human and rodent coding sequences. Five-day old mixed primary cultures from guinea pig myenteric plexus express mRNA corresponding to EDG-1, EDG-3, and EDG-5 [Figure 2.11]. Sequence analysis indicates that these three mRNAs in the guinea pig have a high degree of homology with known nucleotide and amino sequences from human, rat, and mouse [Table 2.1]. Neither EDG-6 nor EDG-8 mRNA were found to be expressed in ENS cultures, yet it remains a possibility that our primers were not of sufficient precision as they were based on incomplete sequence data at the time of this study.

EDG-1 and EDG-3 Expression by Immunocytochemistry

In earlier work, we demonstrated that cells with distinct glial morphology stain positively immunocytochemically for glial fibrillary acidic protein and S-100, specific markers for glia, while staining negatively for fibronectin (fibroblasts) or neuron-specific enolase or MAP2 (neurons) (6). In this investigation, enteric glial cells stained intensely positive for EDG-1 and EDG-3, and weakly positive for EDG-5 [Figure 2.12].

Discussion

The present study demonstrates that myenteric glia possess the necessary signaling processes for transducing responses to a novel class of signaling molecules—sphingolipids. The data supporting this conclusion are four-fold: 1) multiple components of the sphingomyelin cycle (sphingomyelin, sphingomyelinase, sphingosine and sphingosine-1-phosphate) and the related lysosphingolipids and lysoglycerolipids (sphingosylphosphorylcholine and lysophosphatidic acid, respectively) selectively cause calcium signaling in primary cultures of enteric glia; 2) S1P–induced Ca^{2+} signaling is dose-dependent with respect to both the percentage of responding cells and the incremental rise in $[Ca^{2+}]_i$; 3) responsiveness to S1P desensitizes following repetitive exposure; and 4) S1P acts via a PTX-insensitive mechanism to activate PLC and mobilize thapsigargin- and IP_3-sensitive intracellular Ca^{2+} stores, precipitating the influx of extracellular calcium. RT-PCR analysis reveals that cultured cells of the guinea pig myenteric plexus express three members of a new family of sphingolipid-responsive G protein-coupled receptors: EDG-1, EDG-3, and EDG-5. Immunocytochemical analysis

suggests that all three EDG receptors are expressed in myenteric cultures although EDG-5 levels were noticeably lower than those for EDG-1 and EDG-3 in enteric glial cells.

Sphingolipid Signaling

Recent evidence suggests that enteric glia play an active role within the enteric nervous system. Previous reports have shown that enteric glia, like astrocytes of the CNS, undergo Ca^{2+} signaling in response to a variety of neuropeptides and to purinergic compounds such as ATP (6-9). The potential involvement of sphingomyelin-derived lipid compounds as signaling molecules within the ENS suggests that non-purinergic, non-peptidergic mechanisms may also act to modulate glial activity within the myenteric plexus. The finding that S1P is a potent agonist for Ca^{2+} signaling in enteric glial cells, and that a variety of other sphingomyelin metabolites and related lipids also have selective effects in these cells, implies that there may be multiple mechanisms for lipid signaling in the ENS.

Sphingolipid metabolites such as S1P and its receptors have recently been shown to affect a wide variety of biological processes including cellular proliferation, migration and development (30, 39-49), neurite retraction (50, 51), and neuronal survival and differentiation (52). S1P causes Ca^{2+} signaling in cultured CNS astrocytes and Ca^{2+} transients and shape changes in C6 glioma cells (24). There is no established role for sphingolipids within the enteric nervous system. This study provides the first evidence for sphingolipid signaling in cultured enteric glial cells, which share many morphological and functional similarities with their CNS counterparts.

A common feature of many studies of S1P is the ability of this compound to increase intracellular calcium levels. Two general models have been proposed for Ca^{2+} signals generated by S1P: 1) direct release of $Ca^{2+}{}_i$ stores due to interaction between the lipid and internal Ca^{2+} pools, and 2) increases in $[Ca^{2+}]_i$ as a consequence of a plasma membrane-mediated signaling event (23). This investigation offers strong support for the second mechanism in enteric glial cells.

Goodemote et al. (53) initially observed that S1P effects in Swiss 3T3 fibroblasts were pertussis toxin sensitive, suggesting this lipid possessed activity that resembled other agonists of G-protein-coupled receptors (GPCRs). The recent characterization of a family of GPCRs (EDG receptors) with affinity for S1P and related lipids has established a role for receptor-mediated lipid signaling events. Previously an orphan receptor, EDG-1 was the first putative S1P receptor to be identified (36, 54-56). To date, seven additional EDG receptors have been identified that are differentially activated by S1P, SPC and LPA (57-69).

The variety of sphingolipid signaling molecules that were demonstrated to increase $[Ca^{2+}]_i$ in enteric glia implies that multiple EDG receptors may be present in the ENS. EDG-1, EDG-3, EDG-5/H218/AGR16, EDG-6, and EDG-8 are responsive to S1P (30, 35, 36, 39, 51, 55, 56, 58, 63, 66, 69, 70) and, to a lesser degree, SPC (58, 66, 69, 70). EDG-2/*Vzg*-1, EDG-4, and EDG-7 appear to be selectively sensitive to LPA (57, 59, 60, 62). Using RT-PCR, EDG-1, EDG-3 and EDG-5 receptor mRNAs were demonstrated in cultured cells from guinea pig myenteric plexus, and expression of each of these receptors could be demonstrated immunocytochemically. The expression of EDG-1, EDG-3, and EDG-5 receptors is consistent with the observation of Ca^{2+}

responses to S1P and SPC by enteric glia. Sensitivity of enteric glia to LPA suggests that at least one additional receptor (EDG-2, EDG-4, or EDG-7) is likely to be expressed as well.

S1P-Induced Signaling

This investigation indicates that S1P is a potent agonist for Ca^{2+} signaling in enteric glia, with dose-dependent effects that were observed at concentrations consistent with measurable levels of this compound in serum (71, 72). S1P responsiveness in enteric glia was maintained in Ca^{2+}-free medium and blocked by thapsigargin, implying that initial Ca^{2+} transients are due to release of Ca^{2+}_i stores. Removal of Ca^{2+} from the medium eliminated the sustained "plateau" in Ca^{2+} transients, consistent with influx of Ca^{2+}_e following initial Ca^{2+} release from intracellular stores. Using rat glioma C6 cells, Tas and Koschel (24) showed that S1P evoked Ca^{2+} release from internal stores and Ca^{2+} influx from the external medium.

EDG-1, EDG-3, and EDG-5 have all been demonstrated to couple to G_i, but EDG-3 and EDG-5 are also capable of interacting with G_q in transfected Sf9 and HEK293 cells (73). If guinea pig EDG-3 couples to G_q, S1P-induced $[Ca^{2+}]_i$ transients would be anticipated to be insensitive to PTX exposure. In many cell stypes, G_q proteins link GPCRs with PLC. The current studies convincingly demonstrate that S1P-induced calcium signaling is antagonized by the PLC inhibitor U73122, but not by the inactive analog, U73343. Other investigators have also reported that S1P signaling involves PLC

(74, 75). Based on experiments utilizing 2APB, a selective antagonist of the IP_3 receptor, extracellular S1P was demonstrated to trigger IP_3-mediated Ca^{2+} signaling.

Intracellular Signaling

While these studies suggest a role for sphingolipid processing in enteric glia, they do not exclude Ca^{2+} signaling by sphingolipid metabolites acting intracellularly. Sphingomyelin, a normal constituent of cellular membranes, induced Ca^{2+} transients in a small percentage of enteric glia. Exposure of cultured glial cells to exogenous administration of sphingomyelinase also caused them to undergo Ca^{2+} signaling. The dose utilized in this investigation (0.1 U/ml) has been previously shown to induce mitogenesis of smooth muscle cells by initiating SM metabolism and producing S1P (76). Although the initial breakdown product of SM—ceramide—has been demonstrated to cause IP_3-mediated intracellular Ca^{2+} release in Xenopus oocytes (77), it had no effect in enteric glia. By utilizing a truncated form of ceramide (C2-CER), which retains biological activity yet is less likely to be further metabolized, it appears that the Ca^{2+} transients initiated by proximal components of the sphingomyelin cycle are attributable to distal products of catabolism: sphingosine or S1P. This experiment addresses the concern that sphingolipid-induced Ca^{2+} signaling in enteric glia is due to non-specific detergent effects by showing that not all lipids elicit cytoplasmic Ca^{2+} changes.

D-erythro-sphingosine caused Ca^{2+} signaling in enteric glia, but its inactive enantiomer (L-erythro-sphingosine) was functionally inert in this experimental model. Several investigations have shown that the activity of S1P within cells is dependent upon

its production by an endoplasmic reticulum-bound sphingosine kinase (26, 40, 76, 78-82). Sphingosine kinase stimulation causes phosphorylation of endogenous sphingosine, producing S1P, which is believed to function by directly mobilizing intracellular Ca^{2+} (26, 80). While other studies have shown S1P—not sphingosine—serves as a $Ca^{2+}{}_i$-mobilizing second messenger, we did not directly demonstrate that sphingosine caused Ca^{2+} signaling only through its intracellular conversion to S1P.

The establishment of a physiological source of S1P has been elusive. Two recent studies suggest that upon activation with prothrombotic stimuli, platelets release amounts of S1P that increase serum levels to around 500 nM (71, 72). While physiological levels of S1P are not known within the gastrointestinal tract, circulating quantities such as those described by Yatomi et al. (72) are similar to concentrations used in these studies. The recruitment and activation of platelets during an inflammatory response within the gastrointestinal tract could serve as a means of producing local concentrations of S1P that would affect enteric glia. Alternatively, if enteric glia possess the ability to process sphingolipids and produce bioactive lipids like S1P, then they may regulate local responses to inflammation.

In conclusion, the data in the current studies show that enteric glial cells are selectively responsive to members of the sphingolipid family of molecules and related compounds. The mechanism of S1P signaling is PTX-insensitive and involves mobilization of $Ca^{2+}{}_i$ stores through the activation of PLC and subsequent production of IP_3. Three S1P receptors (EDG-1, EDG-3 and EDG-5) appear to be expressed in glial cells of the ENS. In addition to responsiveness to peptidergic and purinergic signals, enteric glia possess a signal transduction system responsive to bioactive lipids. While the

functional significance of such a system is currently undefined, these findings represent the first evidence of lipid-mediated signaling events in the ENS and suggest that a number of mechanisms exist for information transfer within the GI tract.

Acknowledgments

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Figures

Figure 2.1. Summary of sphingomyelin and phosphatidylcholine metabolism.

The three bioactive lipids sphingosylphosphorylcholine, sphingosine-1-phosphate, and lysophosphatidic acid are shown in blue. The pertinent enzymes are shown in red.

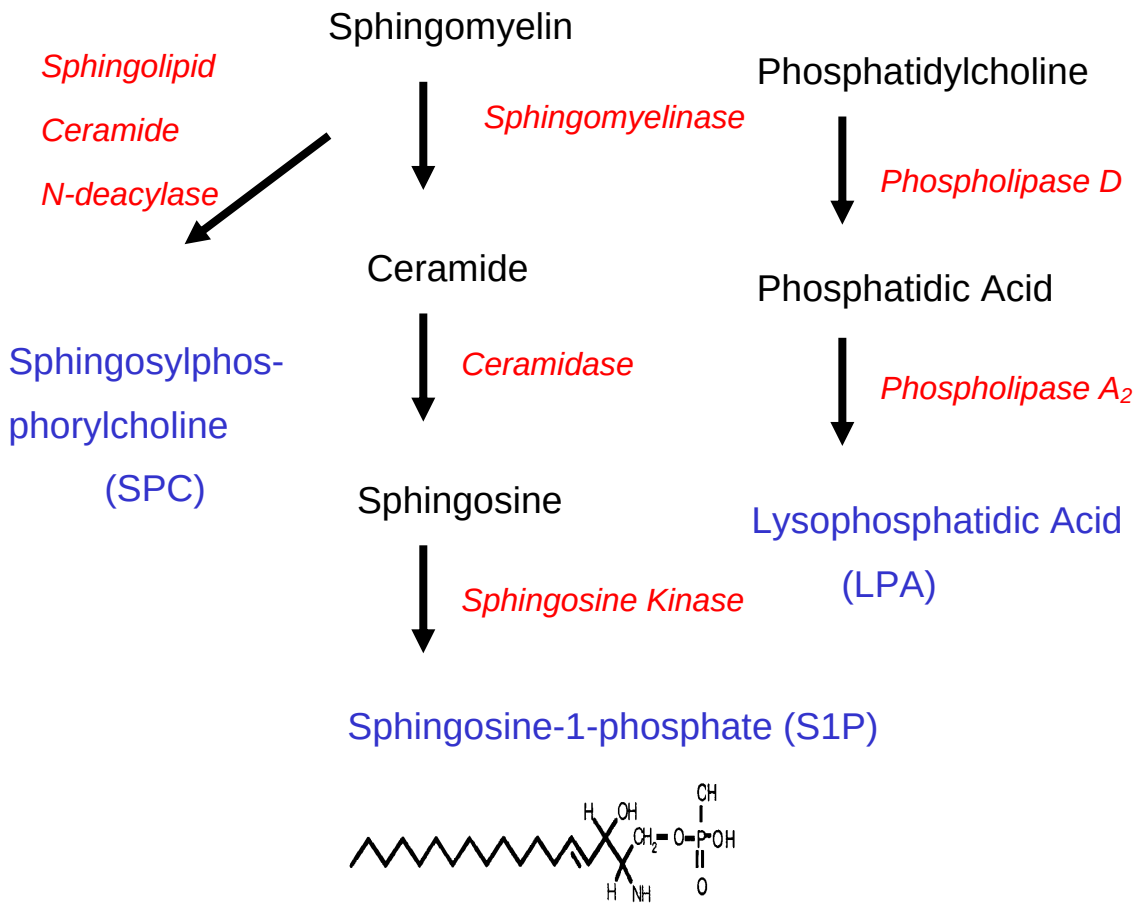
Sphingomyelin
Sphingolipid
Ceramide
N-deacylase
Sphingomyelinase
Phosphatidylcholine
Phospholipase D
Ceramide
Phosphatidic Acid
Sphingosylphos-
phorylcholine
(SPC)
Ceramidase
Phospholipase A2
Sphingosine
Lysophosphatidic Acid
(LPA)
Sphingosine Kinase
Sphingosine-1-phosphate (S1P)
H
OH
CH2
O
P
OH
CH
O
H
NH

FIGURE 2.1

Figure 2.2. Ca^{2+} signaling by bioactive lipids in enteric glia. Sphingomyelin (SM), sphingomyelinase (SMase), D-erythro-sphingosine (D-e-SPH), sphingosine (S1P), lysophosphatidic acid (LPA), and sphingosylphosphorylcholine (SPC) activate Ca^{2+} signaling at the indicated doses whereas C2-ceramide (C2-CER) and L-erythro-sphingosine (L-e-SPH) had no effect. $\Delta[Ca^{2+}]_i$ represents the incremental rise in intracellular calcium concentration (i.e., the difference between resting and stimulated cytosolic $[Ca^{2+}]$) for all responding cells. Each compound was superfused for 300 s; individual cells were exposed to only one agonist. For each condition n ranged from 96 to 184 cells (total number of glia examined was 1187). Data represent mean±SEM for each cell for the $\Delta[Ca^{2+}]_i$ response and for run averages when determining the percentage of cells responding.

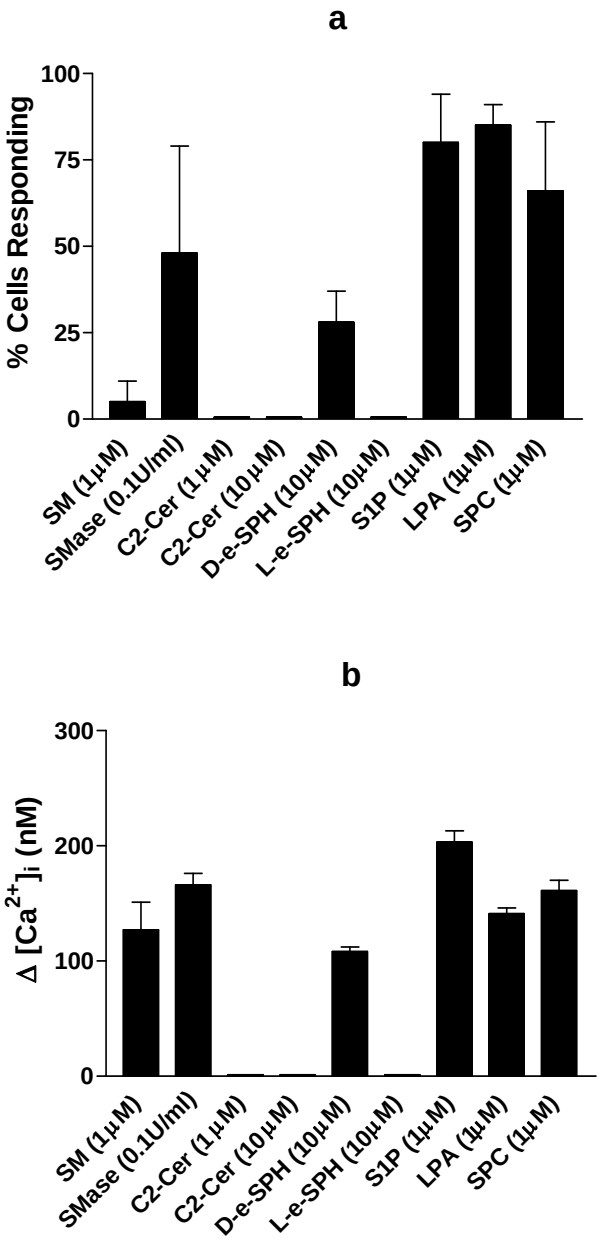
a
% Cells Responding
100
75
50
25
0
SM (1µM)
SMase (0.1U/ml)
C2-Cer (1µM)
C2-Cer (10µM)
D-e-SPH (10µM)
L-e-SPH (10µM)
S1P (1µM)
LPA (1µM)
SPC (1µM)
b
Δ [Ca2+]i (nM)
300
200
100
0
SM (1µM)
SMase (0.1U/ml)
C2-Cer (1µM)
C2-Cer (10µM)
D-e-SPH (10µM)
L-e-SPH (10µM)
S1P (1µM)
LPA (1µM)
SPC (1µM)

FIGURE 2.2

Figure 2.3. Sphingosine-1-phosphate causes robust Ca^{2+} signaling in glial cells of the ENS. Representative tracing following treatment with S1P (1 μM) for 120 s.

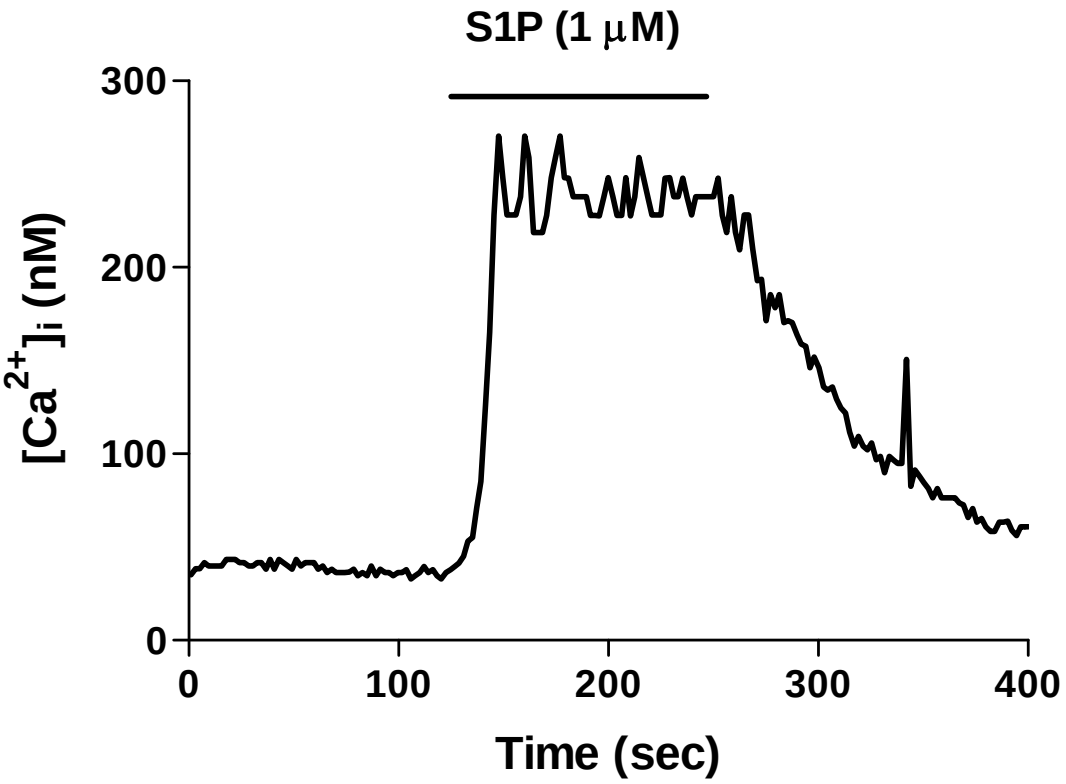

FIGURE 2.3

Figure 2.4. S1P effects on calcium signaling are specific for glial cells in the ENS. Representative tracings following treatment with S1P (1 μM) for 120 s as depicted for an enteric neuron (dashed line) and an enteric glial cell (solid line). In addition to being distinguished from enteric neurons morphologically, enteric glia are not typically responsive to depolarization with 55 mM KCl, as shown.

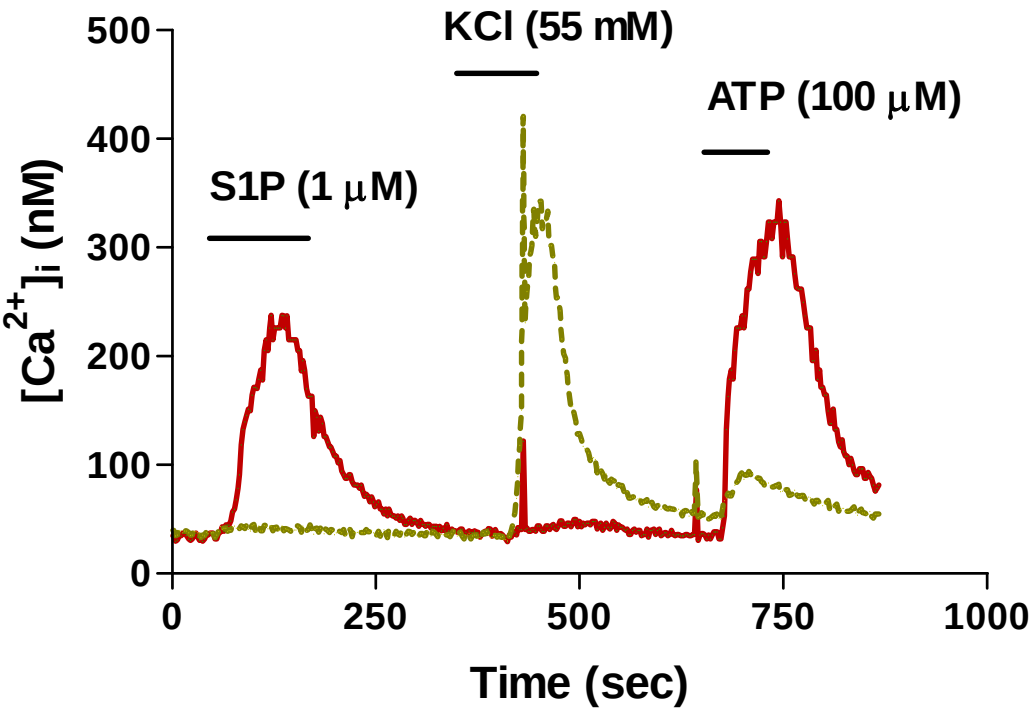
KCl (55 mM)
ATP (100 μM)
S1P (1 μM)
500
400
300
200
100
0
[Ca2+]i (nM)
0
250
500
750
1000
Time (sec)

FIGURE 2.4

Figure 2.5. Concentration-dependent increases in Ca^{2+} signaling evoked by S1P in enteric glia. S1P at the indicated doses activated Ca^{2+} signaling that was dose-dependent with respect to the percentage of responding cells (A) and the incremental rise in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), (B). Each compound was superfused for 120 s; individual cells were exposed to only one agonist. For each condition n ranged from 79 to 164 cells (total number of glia examined was 801). Data represent mean±SEM. *P < 0.05 versus basal by Student's t test.

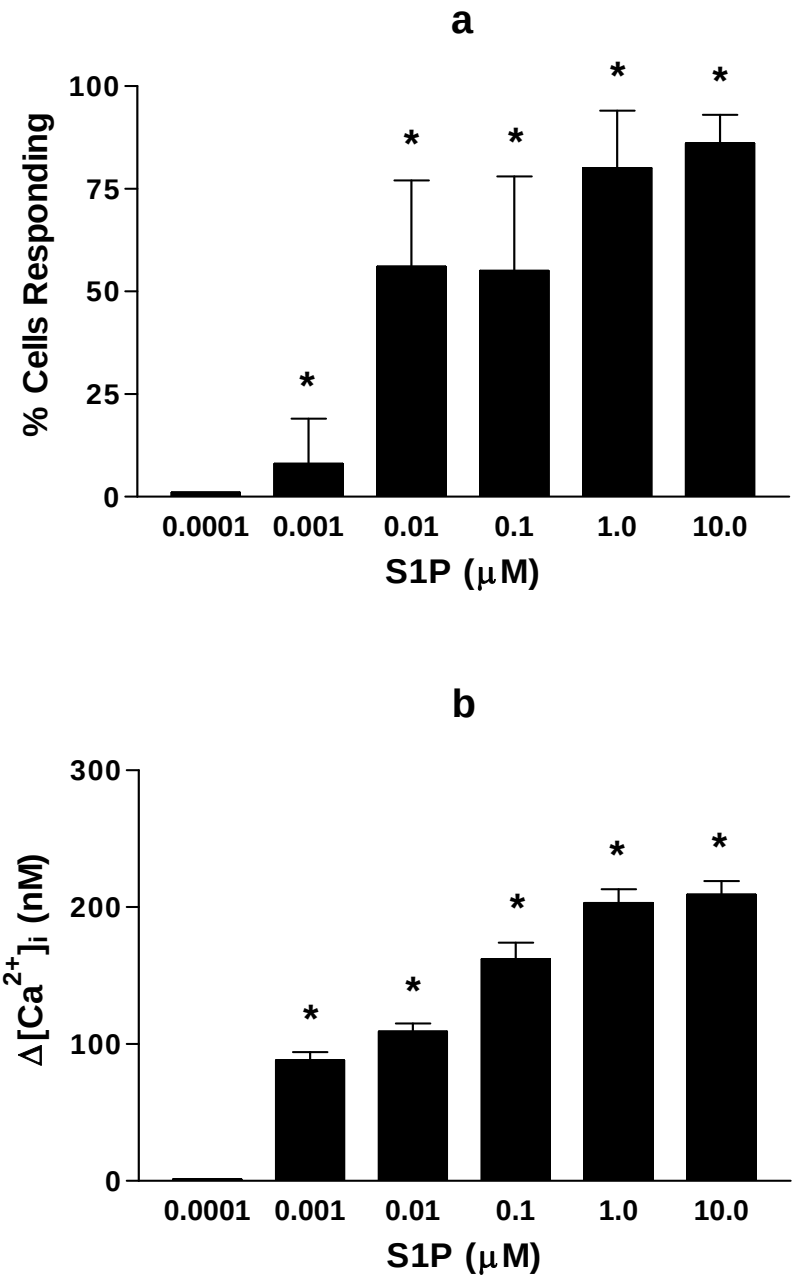
a
% Cells Responding
100
75
50
25
0
0.0001
0.001
0.01
0.1
1.0
10.0
S1P (μM)
b
Δ[Ca2+]i (nM)
300
200
100
0
0.0001
0.001
0.01
0.1
1.0
10.0
S1P (μM)

FIGURE 2.5

Figure 2.6. Desensitization following repetitive S1P exposure. Repetitive superfusion of enteric glia with S1P (1 μM, 60 s) in calcium-containing buffer, separated by 250 s of buffer perfusion, evoked decreasing peak $[Ca^{2+}]_i$ responses (A). Subsequent treatment with ATP (100 μM) generates a large Ca^{2+} signal, indicating diminished responsiveness to S1P is not attributable to internal Ca^{2+} store depletion. This tracing is typical of 116 glial responses. B. Compiled data from all generated Ca^{2+} signals. Data represent mean±SEM. $*P < 0.05$ versus initial response (Peak I) by Student's *t* test.

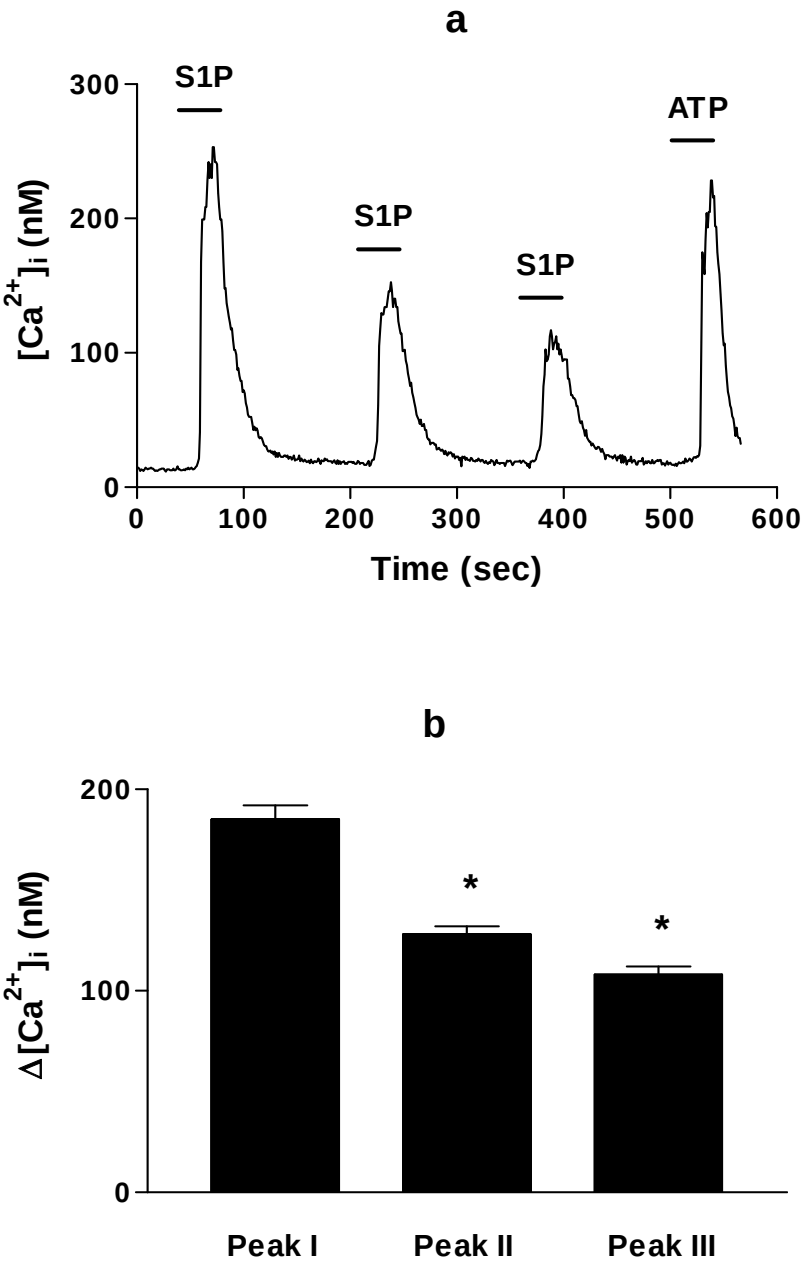
a
S1P
S1P
S1P
ATP
300
200
100
0
[Ca2+]i (nM)
0
100
200
300
400
500
600
Time (sec)
b
200
100
0
Δ[Ca2+]i (nM)
*
*
Peak I
Peak II
Peak III

FIGURE 2.6

Figure 2.7. S1P signals involve intracellular and extracellular Ca^{2+}. Removal of extracellular Ca^{2+} from the perfusion buffer had no effect on initial peak responses to S1P (1 μM, 120 s) but abolished the sustained plateau found in control conditions. (n=161 and 100 for Ca^{2+}-containing and Ca^{2+}-free conditions, respectively.)

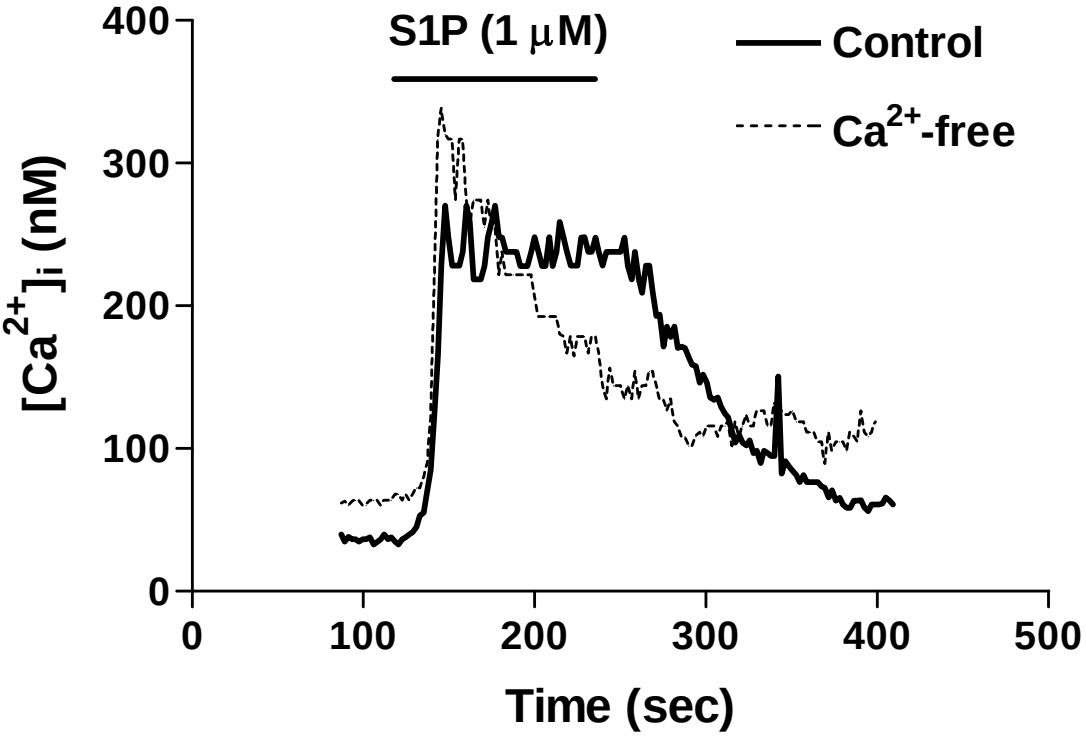
S1P (1 μM)
Control
Ca2+-free
[Ca2+]i (nM)
400
300
200
100
0
0
100
200
300
400
500
Time (sec)

FIGURE 2.7

Figure 2.8. Depletion of intracellular Ca^{2+} (Ca^{2+}_i) stores blocks S1P responses.

Superfusion of thapsigargin (1 μM), an endoplasmic reticulum Ca^{2+}-ATPase inhibitor, for 300 s in Ca^{2+}-free buffer causes Ca^{2+} fluxes in enteric glia by preventing refilling of Ca^{2+}_i stores. Subsequent exposure to S1P (1 μM, 120 sec) produced no $[Ca^{2+}]_i$ response in these cells. n=123 and 90 for control and experimental groups, respectively. Data represent mean±SEM. *$P < 0.05$ versus control.

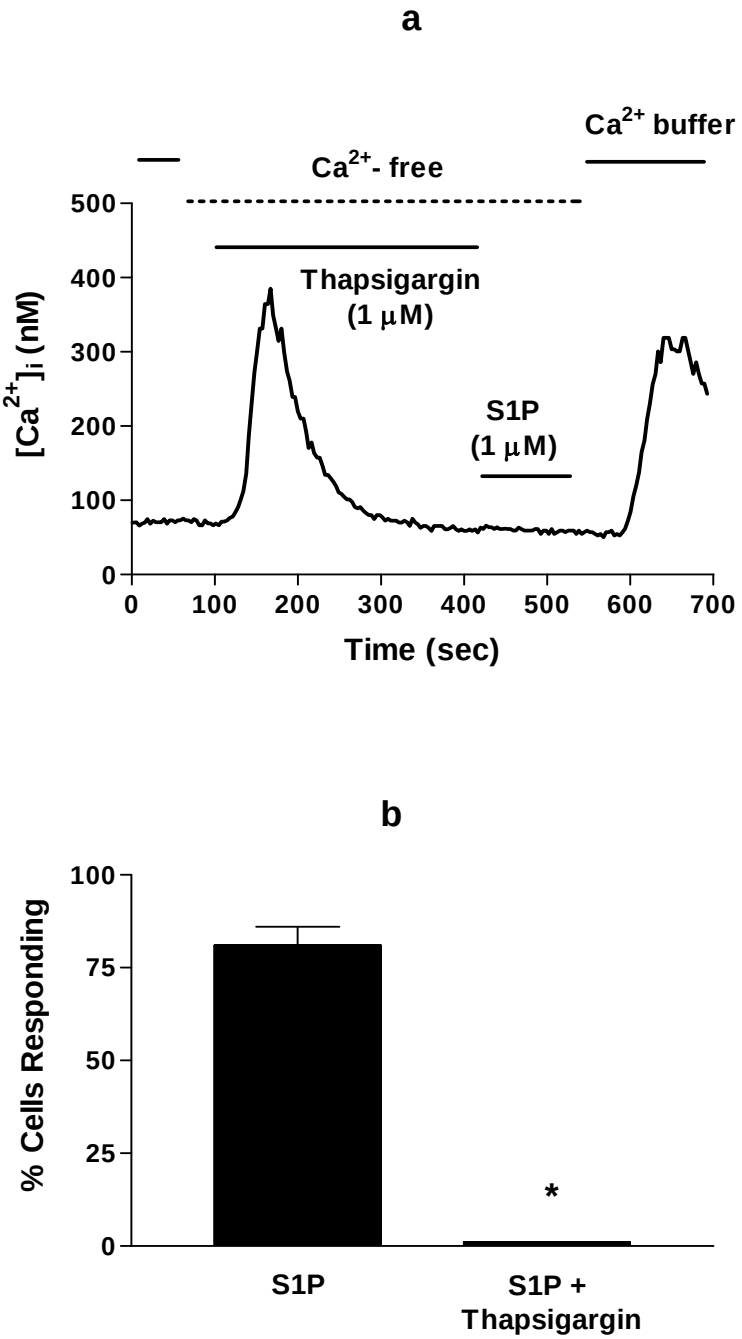
a
Ca2+ buffer
Ca2+- free
Thapsigargin
(1 μM)
S1P
(1 μM)
[Ca2+]i (nM)
500
400
300
200
100
0
0
100
200
300
400
500
600
700
Time (sec)
b
% Cells Responding
100
75
50
25
0
*
S1P
S1P +
Thapsigargin

FIGURE 2.8

Figure 2.9. Phospholipase C (PLC) activation by S1P. Enteric glia were treated with either the PLC inhibitor U73122 (1 μM) or its inactive analog U73343 (1 μM) before exposure to S1P (1 μM). (n=145, 171, and 170 for control, U73122-treated, and U73343-treated groups, respectively.) Data represent mean±SEM. *$P < 0.05$ versus control.

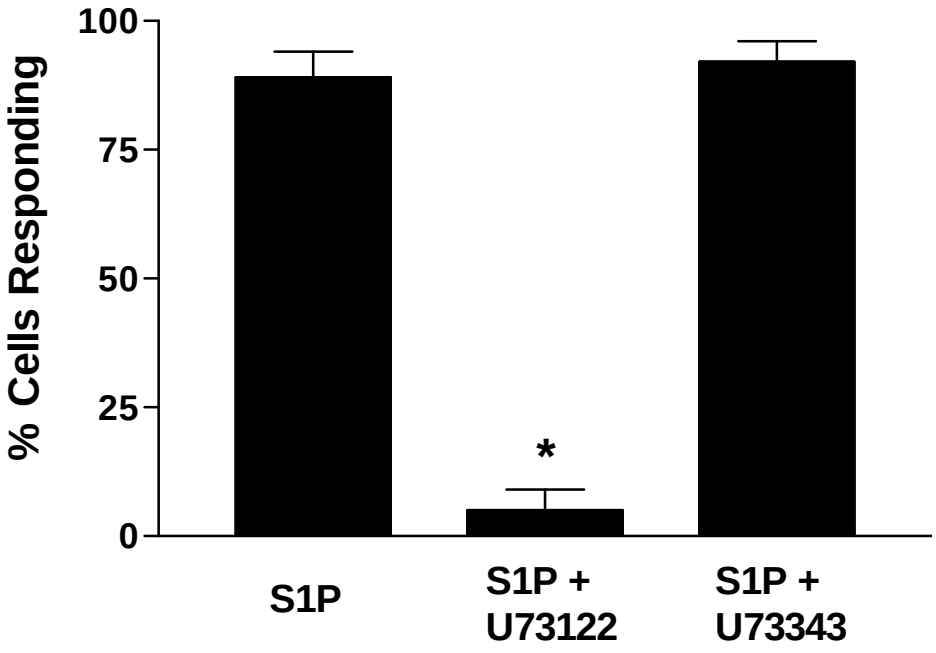

FIGURE 2.9

<u>Figure 2.10.</u> S1P-induced Ca^{2+}_i mobilization involves the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R). The cell-permeant IP_3R inhibitor 2APB dose-dependently reduced S1P responses in enteric glia. For each condition n ranged from 126 to 195 cells (total number of glia examined was 770). Data represent mean±SEM. **P* < 0.05 versus control.

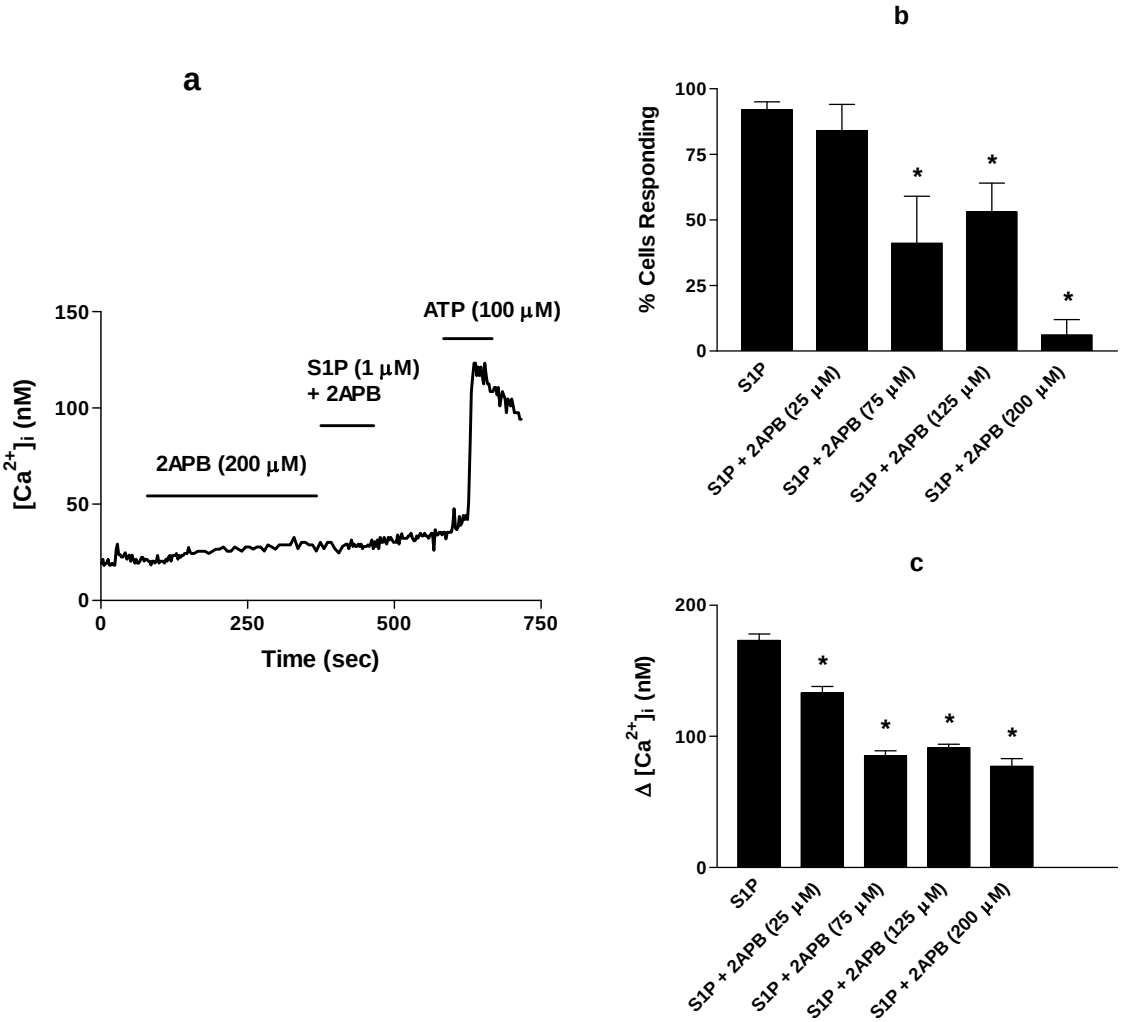
a
150
100
50
0
[Ca2+]i (nM)
2APB (200 μM)
S1P (1 μM)
+ 2APB
ATP (100 μM)
0
250
500
750
Time (sec)
b
100
75
50
25
0
% Cells Responding
*
*
*
S1P
S1P + 2APB (25 μM)
S1P + 2APB (75 μM)
S1P + 2APB (125 μM)
S1P + 2APB (200 μM)
c
200
100
0
Δ [Ca2+]i (nM)
*
*
*
*
S1P
S1P + 2APB (25 μM)
S1P + 2APB (75 μM)
S1P + 2APB (125 μM)
S1P + 2APB (200 μM)

FIGURE 2.10

Figure 2.11. S1P-responsive G protein-coupled endothelial differentiation gene (EDG) receptor mRNAs EDG-1, EDG-3 and- EDG-5 are expressed in the enteric nervous system. RT-PCR products of RNA extracted from 5 d old myenteric plexus primary cell cultures corresponding to EDG receptor coding sequences are shown in lanes 2-4: EDG-1 (892 bp), EDG-3 (715 bp), and EDG-5 (667 bp). Lanes 1 and 6 correspond to molecular size markers. Lane 5 represents the negative control (RT-PCR performed without extracted RNA).

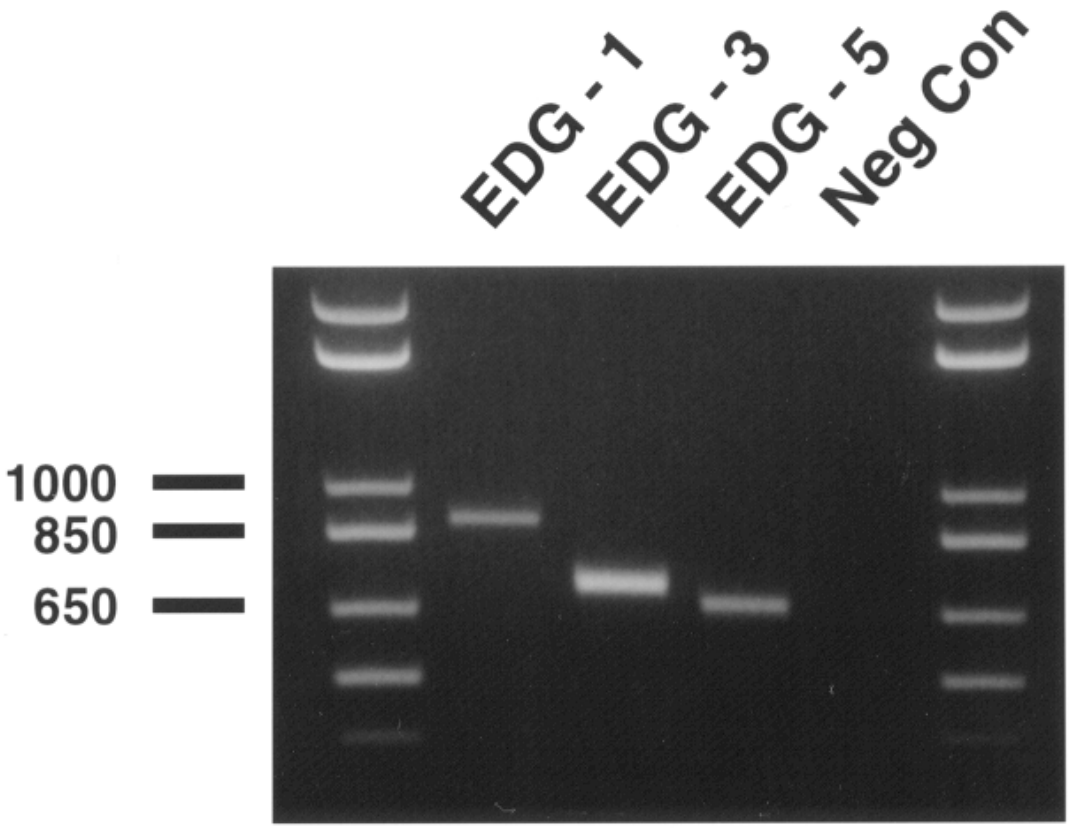

FIGURE 2.11

Table 2.1. Percent homology of guinea pig (Cavia porcellus) EDG-1, EDG-3, EDG-5 sequences to other species. Information based upon comparison of guinea pig EDG-1 (876 bp, Genbank accession number AF289990), EDG-3 (689 bp, Genbank accession number AF289991), and EDG-5 (607 bp, Genbank accession number AF289992) with known nucleotide and amino acid sequences of other species found in Genbank.

	DNA	Protein
EDG-1		
Human	87%	95%
Mouse	87%	95%
Rat	87%	95%
EDG-3		
Human	87%	91%
Mouse	87%	90%
Rat	88%	92%
EDG-5		
Human	88%	91%
Mouse	86%	89%
Rat	83%	88%

TABLE 2.1

Figure 2.12. Immunocytochemical staining for EDG-1, EDG-3 and EDG-5.

Myenteric plexus cultures from neonatal guinea pigs were stained for EDG-1 (A), EDG-3 (C) or EDG-5 (E). Relevant negative controls are represented in panels B, D and F. Myenteric neurons were unstained.

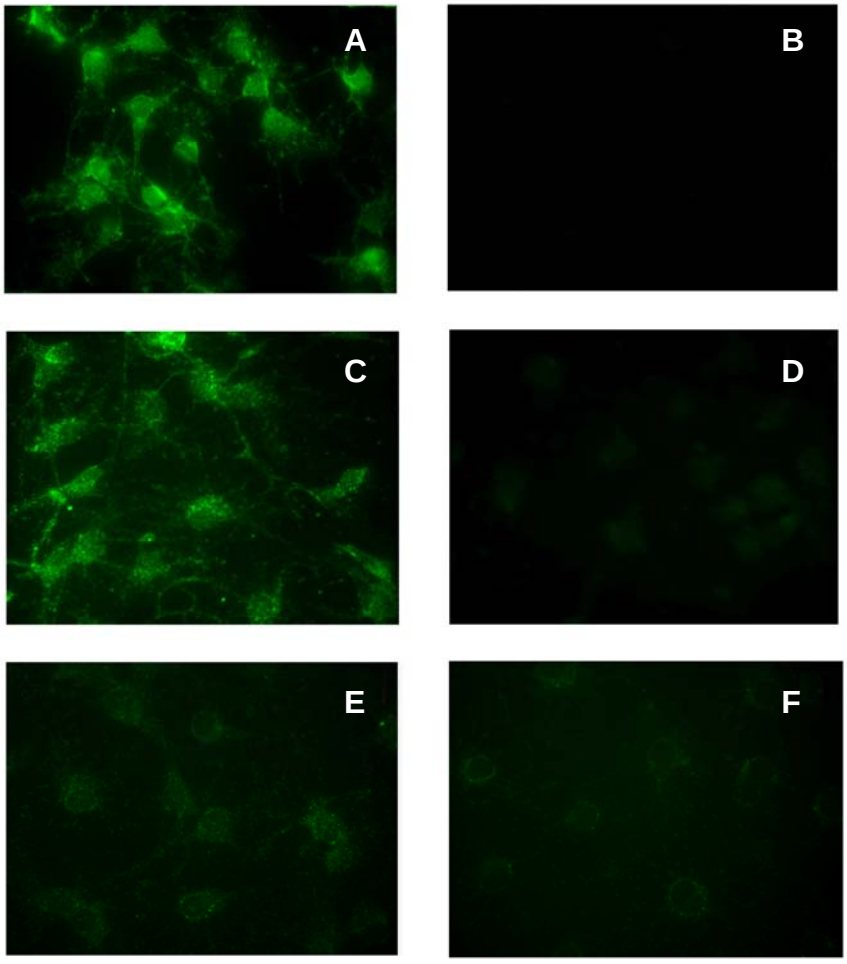

FIGURE 2.12

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CHAPTER 3

LYSOPHOSPHATIDIC ACID STIMULATES CALCIUM TRANSIENTS IN ENTERIC GLIA

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Abstract

The enteric nervous system (ENS) plays an integral role in gastrointestinal tract function. Within this intricate network, enteric glia are crucial in the maintenance of normal bowel integrity, yet their signaling mechanisms are poorly understood. We recently described a role for the novel lipid signaling molecule sphingosine-1-phosphate and its members of the Endothelial Differentiation Gene (EDG) family in the ENS. With the long-term goal of understanding the multiple mechanisms of neurotransmission within the gut, our investigation of bioactive lipids continues with the current studies of a

related lysophospholipid: lysophosphatidic acid (LPA). Here we report that enteric glia, and not enteric neurons, selectively respond to LPA—a product of phosphatidylcholine metabolism. LPA causes dose-dependent calcium (Ca^{2+}) signaling in these cells over a range from 100 pM to 10 μM. Our data suggest that LPA functions via an extracellular receptor in the ENS. The elicited calcium transients involve the mobilization of intracellular Ca^{2+} stores followed by the influx of extracellular Ca^{2+} as LPA signals are obliterated following the depletion of intracellular Ca^{2+} and attenuated by the removal of Ca^{2+}_e from the perfusion buffer. This signal transduction cascade appears to at least partially involve a G protein of the G_i subtype since pretreatment with pertussis toxin reduces the magnitude of LPA transients. Additionally, repetitive exposure yields diminished responsiveness—a hallmark of receptor-mediated desensitization. Inhibition of the inositol 1,4,5-trisphosphate (IP_3) receptor abolishes LPA signals, indicating that the effects of LPA in enteric glia involve the classic route of receptor-mediated Ca^{2+} signaling rather than coupling to a second messenger other than IP_3. Further supporting a receptor-mediated event, RT-PCR analysis demonstrates the presence of two LPA-coupled EDG receptor mRNAs (EDG-2 and EDG-7) in myenteric plexus primary cultures. EDG-2 expression in glial cells of the ENS was confirmed immunocytochemically. Taken together with our previous data on S1P signaling in enteric glia, we conclude that multiple lipid-activated signaling mechanisms exist in these cells. We propose that in addition to established traditional peptidergic and purinergic mechanisms, LPA functions as a novel signaling molecule within the ENS.

Introduction

A family of signaling molecules and growth factors, collectively known as "bioactive lipids", includes sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA). Both are products of membrane lipid catabolism. S1P is enzymatically generated from sphingomyelin whereas LPA is derived from phosphatidylcholine [see Chapter 2, Figure 2.1]. Each lysophospholipid has been implicated in a diverse array of cellular events including cell growth and differentiation, cytoskeletal changes and cell migration, and calcium (Ca^{2+}) signaling (1-5). The Ca^{2+} ion is a critical second messenger which serves to transduce extracellular signals into biological responses. In neural systems, cytosolic levels of Ca^{2+} are an integral factor in gene expression, apoptosis, intercellular communication, synaptic neurotransmission, and long-term potentiation—events in which glia participate (6).

LPA and S1P are distinctive in that, although lipids, they act as extracellular signaling molecules in a receptor-mediated fashion (7). A family of G protein-coupled receptors with affinity for S1P (endothelial differentiation gene receptors: EDG-1, EDG-3, EDG-5, EDG-6, and EDG-8) and LPA (EDG-2, EDG-4, and EDG-7) has been described (8-10). Although the evidence in support of S1P and LPA functioning via extracellular receptors is convincing, the mechanisms responsible for Ca^{2+} mobilization are not completely defined. A role for these bioactive lipids in the enteric nervous system has not been recognized.

We hypothesized that LPA could serve as a signaling molecule within the myenteric plexus and that it would function through putative LPA receptors, EDG-2,

EDG-4, or EDG-7. The current studies of the mechanisms by which LPA affects intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in enteric glia demonstrate that: 1) LPA causes dose-dependent Ca^{2+} signaling; 2) LPA transients involve intracellular and extracellular Ca^{2+}; 3) LPA-induced Ca^{2+} responses desensitize with repetitive exposure; 4) LPA effects are attenuated by pertussis toxin exposure; 5) LPA mobilizes thapsigargin- and inositol-1,4,5-trisphosphate-sensitive intracellular Ca^{2+} stores; and 6) myenteric plexus cultures express EDG-2, and EDG-7 receptor mRNA, with EDG-2 demonstrated immunocytochemically in enteric glia.

Experimental Methods

Materials and Reagents

Lysophosphatidic acid (LPA), adenosine triphosphate (ATP), collagenase type V, thapsigargin, U73122, U73343, trypsin-EDTA, soybean trypsin inhibitor (type I-S), penicillin-streptomycin solution, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), and saponin were from Sigma Chemical (St. Louis, MO). Pertussis toxin (PTX) was from Calbiochem (San Diego, CA). Hanks' balanced salt solution (HBSS), medium 199, and L-glutamine were from GIBCO BRL (Grand Island, NY). Rat tail collagen was from Boehringer Mannheim (Mannheim, Germany). New Serum I (NSI) was from Collaborative Research (Bedford, MA). 2-aminoethoxydiphenyl borate (2-APB) was from Tocris (Ballwin, MO). Fura-2-AM, fluorescein conjugated streptavidin (S-869), DAPI cytological nuclear conterstain kit (C-7590) and prolong antifade kit (p-

7481) were from Molecular Probes, Inc. (Eugene, OR). TRIzol reagent, DNAse I, SuperScript II RNase H^- Reverse Transcriptase (RT), and RT buffer were from Life Technologies, Inc. (Grand Island, NY). Deoxynucleotides (dNTP mix) and oligo d(T) primers were from Roche Molecular Biochemicals (Indianapolis, IN). RNase inhibitor was from Promega (Madison, WI). Advantage cDNA polymerase mix and PCR reaction buffer were from Clontech Laboratories, Inc. (Palo Alto, CA). EDG-2 C-terminus rabbit polyclonal Ab (C174P), EDG-2 blocking peptide (X1269B), and EDG-4 N-termimus mouse monoclonal Ab (C180M) are from Exalpha Biologicals, Inc. (Boston, MA). One-day-old male Duncan-Hartley guinea pigs were obtained from Simonsen Labs (Gilroy, CA).

Myenteric Plexus Isolation

Dispersed primary cultures of guinea pig myenteric plexus were prepared on collagen-coated coverslips and used for experiments within 10 days post-plating using a protocol refined in our laboratory (11). One- to three-day-old Duncan Hartley guinea pigs were sacrificed and their taenia coli were removed using sharp dissection with tissue forceps and Castro-Viejo scissors then placed in Hanks Balanced Salt Solution supplemented with 0.1% Type IV collagenase for 16-24 h at 4^{o}C. After a 30-60 minute incubation at 37^{o}C, the muscle layers of the taenia coli were separated from the myenteric plexus with the use of fine tissue forceps and a dissecting microscope. The myenteric plexus was trypsinized for 30 min at 37^{o}C using 0.1% trypsin-EDTA solution, triturated with siliconized flamed Pasteur pipettes of decreasing diameter, and plated on aseptically-prepared collagen-coated 22 mm glass coverslips in a 35 mm culture dish

using sterile technique. Cultures were exposed to complete medium 199 plus 5% NSI and 0.01% trypsin inhibitor. Penicillin-streptomycin solution was added for the first 48 h at a 2% concentration. Antimitotic agents were not added. Medium was changed every 24-36 hours. The cultures were incubated at 37^{o}C with 5% CO_2. All animals were used in accordance with established guidelines and protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan Medical Center (approval no. 7340).

Solutions

All experiments were performed in standardized solutions except when noted. Standard control buffer was a modified Krebs-Ringer solution at pH 7.40 containing (in mM) 118 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 10 HEPES, 15 $NaHCO_3$, 11 glucose, 0.9 NaH_2PO_4, and 0.8 $MgSO_4$. In Ca^{2+}-free control buffer, the $CaCl_2$ was omitted and 0.5 mM EGTA was added.

Loading and Cell Preparation for Imaging

For loading purposes, fura 2-AM was added to achieve a final concentration of 2 μM for 45 min to cultured myenteric plexus and incubated at 37^{0}C in serum-containing growth media described above that was changed no less than 24 hours prior to avoid acute effects of new serum supplementation. Loaded coverslips were washed and then stored in serum-free control buffer and placed in a lucite superfusion chamber

(approximate volume 0.5 ml). The superfusion rate of the control buffer and experimental solutions was 1 ml/min at 37^{o}C.

Ca^{2+} Measurements

A Zeiss Axiovert inverted microscope and an Attofluor imaging system (Rockville, MD) were used to determine single-cell intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was calculated from the ratios of the fluorescence intensities of fura-2 at 334 and 380 nm, monitored by an intensified charge-couple device camera, and subsequently digitized. Calibration of the system was performed with the two-point standardized equation, using fura-2-free acid:

$$[Ca^{2+}]_i = K_d\,[(R-R_{low})/(R_{high} - R)]b$$

where K_d is the dissociation constant of the Ca^{2+}-fura 2 complex (225), R is F_{334}/F_{380}, i.e., the fluorescence at 334 nm excitation divided by the fluorescence at 380 nm excitation, R_{low} is the ratio at zero Ca^{2+} (1 mM EGTA), R_{high} is the ratio at high Ca^{2+} (1 mM $CaCl_2$), and b is F_{380} (zero Ca^{2+})/F_{380} (saturating Ca^{2+}). Frames were not averaged to obtain images. The system was used to obtain whole field images. Boxes were placed on all cells of interest for simultaneous measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). A ratio pair was taken at every 1.5-3.0 s.

RNA Extraction and Reverse Transcription (RT)

Total cellular RNA was isolated from 5 day-old myenteric plexus primary cell cultures using TRIzol reagent according to manufacturer's directions. Single strand cDNA synthesis was performed as follows: 20 µl of reverse transcription mixture contained 1 µg of DNase I pre-treated total RNA, 0.5 µg of oligo d(T) primer, 4 µl of 5 x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 40 units of RNase inhibitor, and 200 units of reverse transcriptase (SuperScript II RT). The RT reaction was carried out at 42^{o}C for 50 min followed by heat inactivation at 70^{o}C for 15 min.

Polymerase Chain Reaction (PCR)

Fifty µL of PCR reaction mixture contained 1 µl of RT products, 1X cDNA PCR reaction buffer, 400 nM of each primer, 200 µM of dNTP mix, and 1X Advantage cDNA polymerase mix. The PCR was carried out using a Perkin-Elmer Thermal Cycler (Norwalk, CT). Samples were denatured initially at 94^{o}C for 1 min and the PCR was performed as follows: 38 cycles of 35 sec at 94^{o}C, 30 sec at 57^{o}C and 1 min 45 sec at 72^{o}C for EDG-2; 42 cycles of 30 sec at 94^{o}C, 30 sec at 65 ^{o}C and 1 min 30 sec at 72^{o}C for EDG-4; 38 cycles of 35 sec at 94^{o}C, 2 min at 68^{o}C, and 7 min at 72^{o}C for EDG-7, following by the final extension at 72 ^{o}C for 7 min.

PCR primers used for guinea pig mRNA detection were deduced from human, mouse and rat published sequences. The nucleotide sequences of sense and antisense primers with the expected product size are follows: EDG-2, CAT GGG CCA GTG CTA

CTA CAA CGA G (sense) and GCA GCA GAG AAG GCG GCG GAA GGT G (antisense) (927 bp); EDG4, CAT GGG CCA GTG CTA CTA CAA CGA G (sense) and CGG AAG GTG CGG CGC ATC TCA G (antisense) (912 bp); EDG-7, ACT TGC TGG TTA TCG CCG TGG AGA GG (sense) and CTG CTG AGG ACT GTG GAG GGG ATG C (antisense) (634 bp). For negative controls, PCR reactions were performed for each of the primer pairs in the absence of transcript as others have reported.

Sequencing and Data Analysis

PCR products were electrophoretically analyzed on 1.5% agarose gel containing ethidium bromide. DNA bands were excised from the gel and purified as follows: gel slices were crushed in the tube and sample DNA was extracted twice with buffered phenol and once with phenol:chloroform:isoamyl (25:24:1) followed by ethanol precipitation with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol and washing with 70% ethanol. The purified DNA fragments were directly sequenced by the DNA sequencing core at the University of Michigan using Applied Biosystems DNA sequencers. The nucleotide sequences of EDG-2 and EDG-7 have been submitted to GenBank with the accession numbers of AY013708 and AY013709.

Immunochemistry

Cultured guinea pig myenteric plexus cells were used on day 5 or day 6. Cells were fixed in freshly prepared 4% paraformaldehyde/PBS (pH 7.4) for 20 min at 4^{O} C (all

steps were performed at 4^{o}C unless otherwise noted. Following rinsing in PBS, the cells were incubated with blocking/permeabilization buffer (10% normal goat serum, 2% BSA and 0.1% saponin in PBS) for 20 min at room temperature. After washing with PBS, the cells were incubated with primary antibody solution overnight at 4^{o}C. Mouse monoclonal antibody against EDG4 was diluted at 1:50 in PBS with 1.5% goat serum. Rabbit polyclonal antibody against EDG2 was diluted at 1:100 in PBS with 1.5% goat serum. As a negative control, rabbit polyclonal antibodies were pre-incubated with specific blocking peptides for 20 min at 37^{O} C; the sample without primary antibody was used for negative control for mouse monoclonal antibody samples. After thoroughly a wash with PBS, the samples were incubated for 45 min with biotinylated goat anti-mouse or anti-rabbit antibody (1:200 diluted in PBS with 1.5% goat serum), illuminated by reaction with fluorescein conjugated streptavidin, stained with DAPI cytological nuclear conterstain kit and mounted with prolong antifade kit following company instructions. Samples were examined using a Nikon microscope with epifluorescence capabilities and images were recorded using a Spot camera then processed in Adobe photoshop.

Data Presentation and Calculation

Results are expressed as mean ± SEM. Data were analyzed using ANOVA. Significance was accepted as $P < 0.05$ (95% confidence interval).

Dissection techniques, tissue preparation, media, and reagent vendors remained constant throughout the study. In this study, *n* equals the number of glial cells examined. At least three coverslips were used for each experimental condition. All experimental

conditions were examined on glial cells derived from cell preparations performed on at least two different days.

Results have been calculated only from those responding glia having basal $[Ca^{2+}]_i$ levels < 100 nM, a criteria met by > 95% of glial cells. Cells were considered to be responsive if peak $[Ca^{2+}]_i$ was at least 50 nM higher than the baseline value. Glia with a high $[Ca^{2+}]_i$ before any addition of agonist were considered damaged or leaky and were excluded from the study. Only one microscope field was examined per coverslip. Peak $[Ca^{2+}]_i$ was measured as the highest $[Ca^{2+}]_i$ achieved during agonist exposure. $\Delta[Ca^{2+}]_i$ represents the difference between peak and basal $[Ca^{2+}]_i$.

At the time of the experiments, two criteria were used to determine whether cells of interest were glial cells, as opposed to neurons. *1)* Morphology. Myenteric neurons 2-7 days post-plating are compact and phase-bright, with few or no processes. Enteric glia have a larger, dense nucleus with wide surrounding cytoplasm. *2)* KCl depolarization. At the end of each experiment, the coverslip was superfused with 55 mM KCl. Enteric glia typically do not exhibit increments in $[Ca^{2+}]_i$ on exposure to 55 mM KCl.

Experimental Results

Lysophosphatidic Acid (LPA) Dose-Dependently Causes Ca^{2+} Signaling in Enteric Glia.

Perfusion of glial cells with LPA (1 μM) for 120s caused Ca^{2+} mobilization in nearly 100 % of glial cells (n=394) [Figure 3.1*a*]. LPA produced dose-dependent

increments in the percentage of cells responding [Figure 3.1*b*] and in the elicited $[Ca^{2+}]_i$ increments [Figure 3.1*c*] over the range of 100 pM to 10 μM, with the EC50 of 86 nM. For remaining experiments, 1 μM LPA was chosen as a suitable working dose.

LPA-Mediated Ca^{2+} Signaling Desensitizes with Repetitive Exposure.

Repetitive exposure to LPA produced progressive decrements in peak $[Ca^{2+}]_i$ in enteric glia [Figure 3.2*a*]. An approximately 25% decrease in peak $[Ca^{2+}]_i$ responses was observed between first and second LPA exposures, and another decrement of 30% between the second and third exposures [Figure 3.2B]. 92% of cells responded to LPA and 13% completely desensitized (n=176). To demonstrate that the effect was not due to depletion of intracellular Ca^{2+} stores, cells were subsequently perfused with ATP (100 μM) at the end of each experiment and typical increments in $[Ca^{2+}]_i$ were achieved. The ATP-induced increment in $[Ca^{2+}]_i$ after repetitive exposure to LPA was comparable to the responsiveness to the initial ATP perfusion (455±38, n=301 vs. 469±57 nM, n=117, P>0.05)

LPA Responses are Pertussis Toxin (PTX)-Sensitive.

To investigate the involvement of a PTX-sensitive G protein, enteric glia were preincubated with PTX at 100 ng/ml for 24 h, a concentration and preincubation time found to be inhibitory in other studies. The percentage of cells responding to LPA was significantly different for these two groups: 56% vs. 94% for PTX-treated and non PTX-

treated cells, respectively [Figure 3.3*a*]. Peak $[Ca^{2+}]_i$ generated by LPA in glia pre-exposed to PTX was 95±20 nM (n=138), relative to the peak $[Ca^{2+}]_i$ evoked in control glia of 168±17 nM (n=172) [Figure 3.3*b*].

LPA Mobilizes Intracellular Ca^{2+} Stores and Activates Extracellular Ca^{2+} Influx.

To evaluate the role of intracellular Ca^{2+} (Ca^{2+}_i) stores in LPA-generated calcium signaling, responsiveness of enteric glia to LPA was investigated using Ca^{2+}-free medium supplemented with 1 mM EGTA. Exposure to LPA in a nominally Ca^{2+}-free medium had a minimal effect on the percentage of cells responding or on peak $[Ca^{2+}]_i$. The morphology of the observed Ca^{2+} transients, however, was distinctly different [Figure 3.4*a*]. The absence of extracellular Ca^{2+} (Ca^{2+}_e) abolished the sustained plateau in intracellular $[Ca^{2+}]$, indicating that an influx of Ca^{2+}_e occurs following initial Ca^{2+}_i mobilization. Thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, depletes Ca^{2+}_i stores without generating IP_3. During superfusion with thapsigargin (1 μM) in a Ca^{2+}-free medium, all cells (n=113) responded with increases in $[Ca^{2+}]_i$, followed by return of $[Ca^{2+}]_i$ to baseline; at this point, Ca^{2+}_i stores were presumed to be depleted. Subsequent exposure to LPA produced Ca^{2+} transients in none of the glial cells examined [Figure 3.4*b*]. At the end of each experiment, perfusion with Ca^{2+}-containing buffer induced capacitative calcium entry.

LPA-Induced Ca^{2+}_i Mobilization Involves the Inositol 1,4,5-Trisphosphate (IP_3) Receptor (IP_3R).

The role of IP_3-sensitive intracellular Ca^{2+} stores in LPA signaling was investigated using the membrane-permeable IP_3R antagonist 2APB. 2APB has been utilized in a variety of cell types to reversibly modulate IP_3R activity independent of IP_3 production and binding, voltage-gated calcium channels, or ryanodine-sensitive calcium stores. In this investigation, 2APB had no effect on basal cytosolic $[Ca^{2+}]_i$. Following pretreatment with 2APB (200 μM) for 5 min, only 21% of glia (n=135) responded to LPA exposure vs. 89% for controls (n=141) [Figure 3.5].

EDG-2 And EDG-7 mRNAs are Expressed in the ENS.

To further assess the participation of LPA-responsive G protein-coupled receptors of the endothelial differentiation gene family, RT-PCR analysis was performed using primers generated from known human and rodent coding sequences. Five day old mixed primary cultures from guinea pig myenteric plexus express mRNA corresponding to EDG-2 and EDG-7; mRNA corresponding to EDG-4 was not detected [Figure 3.6]. Sequence analysis indicated that these mRNAs in the guinea pig have a high degree of homology with known nucleotide and amino sequences from human, rat, and mouse.

In prior studies, we have demonstrated that cells with distinct glial morphology stain positively immunocytochemically for glial fibrillary acidic protein and S-100, while staining negatively for fibronectin (fibroblasts) or neuron-specific enolase or MAP2

(neurons). The current study revealed that enteric glial cells stain strongly for EDG-2 but only display trace positivity for EDG-4 [Figure 3.7]. Enteric neurons stained negative for EDG-2 receptor antibody.

Discussion

The current study demonstrates that enteroglial cells from the guinea pig myenteric plexus respond to lysophosphatidic acid via EDG-2 receptors. The findings that support this conclusion are five-fold: 1) LPA produces increments in $[Ca^{2+}]_i$ which are dose-dependent and desensitize with repetitive exposure; 2) responsiveness to LPA is sensitive to pertussis toxin exposure; 3) LPA mobilizes intracellular Ca^{2+} stores that are released via a mechanism involving IP_3 receptor activation; 5) RT-PCR analysis of guinea pig myenteric plexus reveals production of EDG-2 and EDG-7 mRNA, with EDG-2 receptors detected immunocytochemically in enteroglial cells.

Enteric glia outnumber enteric neurons by 2:1 and share morphological similarities to CNS astrocytes; they have irregular shapes, express glial fibrillary acidic protein and do not synthesis a basal lamina (12). Recent reports have demonstrated that astrocytes are active participants in CNS synaptic transmission, in addition to serving supportive and nutritive functions. Enteric glial cells may also exhibit functional properties similar to CNS astrocytes. Enteric glia, like astrocytes, undergo Ca^{2+} signaling in response to purinergic compounds and to a variety of neuropeptides (11, 13, 14). The finding that lysophosphatidic acid is a potent agonist for Ca^{2+} signaling in enteric glial cells suggests that non-purinergic, non-peptidergic mechanisms may also regulate glial

activity within the myenteric plexus. The ability of LPA, a bioactive lipid derived from phosphatidylcholine, to activate myenteric glia has not been previously reported.

Lysophosphatidic Acid and EDG Receptors

The recent characterization of a family of G-protein-coupled receptors with affinity to bioactive lipids has established a role for receptor-mediated lipid signaling mechanisms. The Endothelial Differentiation Gene-1 (EDG-1) receptor was the first member of this group recognized, with sphingosine-1-phosphate (S1P) as its ligand (8, 15). Currently, seven additional EDG receptors have been reported. The varying EDG receptors are differentially activated by S1P, sphingosylphosphorylcholine (SPC) and LPA. EDG-1, EDG-3, EDG-5, EDG-6 and EDG-8 are selective for S1P, and, to a lesser degree, to SPC; EDG-2, EDG-4 and EDG-7 are sensitive to LPA (8-10). Using RT-PCR, EDG-2 and EDG-7 mRNA was detected in the mixed cellular culture of guinea pig myenteric plexus, while EDG-4 was not identified. Myenteric cells with the morphological and immunostaining characteristics of enteroglia were demonstrated to express EDG-2 immunopositivity, consistent with responsiveness to extracellularly applied LPA. Although enteric neurons are also present in these cultures, two observations indicate that enteric neurons do not contribute to glial Ca^{2+} response to LPA: (1) enteric neurons did not respond to LPA with Ca^{2+} transients; (2) immunocytochemical staining showed that enteric neurons stained negative for EDG-2 receptor antibody.

LPA Signaling

A common feature of LPA signaling in many cell types is the ability of this compound to increase intracellular calcium levels. In Tag-Jurkat T cells transiently transfected with human EDG-2 cDNA, extracellular LPA stimulated an initial increase in $[Ca^{2+}]_i$ followed by a more prolonged influx of extracellular Ca^{2+} (16). A similar general mechanism was demonstrated in enteric glial cells. In the current study, LPA responsiveness in enteric glia was maintained in Ca^{2+}-free media and was blocked by thapsigargin, implying that initial Ca^{2+} transients are due to release of $Ca^{2+}{}_i$ stores. Removal of Ca^{2+} from the medium eliminated the sustained plateau in Ca^{2+} transients, consistent with influx of $Ca^{2+}{}_e$ following initial Ca^{2+} release from intracellular stores.

EDG receptors responsive to LPA have been shown to couple to at least three G-proteins: G_q, G_i, and $G_{12/13}$ (17). When recombinant human EDG-2 was stably transfected into rat HTC4 hepatoma cells, LPA-induced mobilization was almost completely blocked by pertussis toxin, indicating mediation via G_i (16). Similarly, in MG63 human osteosarcoma cells, LPA-stimulated activation of Erk1/2 was G_i-dependent (18). In contrast, Ishii and colleagues have reported that mouse EDG-2 (LP_{A1}) can couple to multiple G-proteins to induce LPA-dependent inositol phosphate production, MAP kinase activation or arachidonic acid release (18). In the current study, pretreatment of enteric glial cells with pertussis toxin reduced LPA-stimulated $[Ca^{2+}]_i$ transients by nearly 50%, consistent with involvement of G_i. The action of 2APB, an inhibitor of the IP_3 receptor, strongly suggests the LPA-induced Ca^{2+} mobilization is dependent upon IP_3 generated by phospholipase C activation. Decreasing responsiveness

upon repetitive exposure to LPA is consistent with the event mediated by G-protein-coupled receptors.

A Potential Role for LPA in the ENS

The physiological source of LPA within the enteric nervous system is unknown. LPA is produced as a result of cleavage of membrane-associated phospholipids by phospholipase A2. Activated platelets are an abundant source of extracellular LPA, and serum concentrations in the micromolar range, substantially higher than those used in the current study, have been reported (19). The recruitment and activation of platelets to an area of vascular injury could potentially expose myenteric plexus cells to LPA. Alternatively, if enteric glial cells, or other cellular componenets of the enteric nervous system, possess the ability to release bioactive lipids, these cells might also locally regulate inflammatory responses.

In summary, the current study demonstrates that enteric glial cells respond to LPA and that such responses may be mediated by EDG-2 receptors. The resulting Ca^{2+} signal is pertussis toxin-sensitive and involves mobilization of intracellular Ca^{2+} stores through activation of production of IP_3.

Acknowledgments

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Figures

<u>Figure 3.1.</u> Concentration-dependent increases in Ca^{2+} signaling evoked by LPA in enteric glia. Lysophosphatidic Acid (LPA) (1μM) activates Ca^{2+} signaling in enteroglial cells (A). Myenteric neurons were unresponsive to LPA. LPA at the indicated doses activated Ca^{2+} signaling that was dose-dependent with respect to the percentage of responding cells (B) and the incremental rise in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), (C). LPA was superfused for 120 s; individual cells were only exposed to LPA once. For each condition *n* ranged from 95 to 182 cells (total number of glia examined was 719). Data represent mean±SEM.

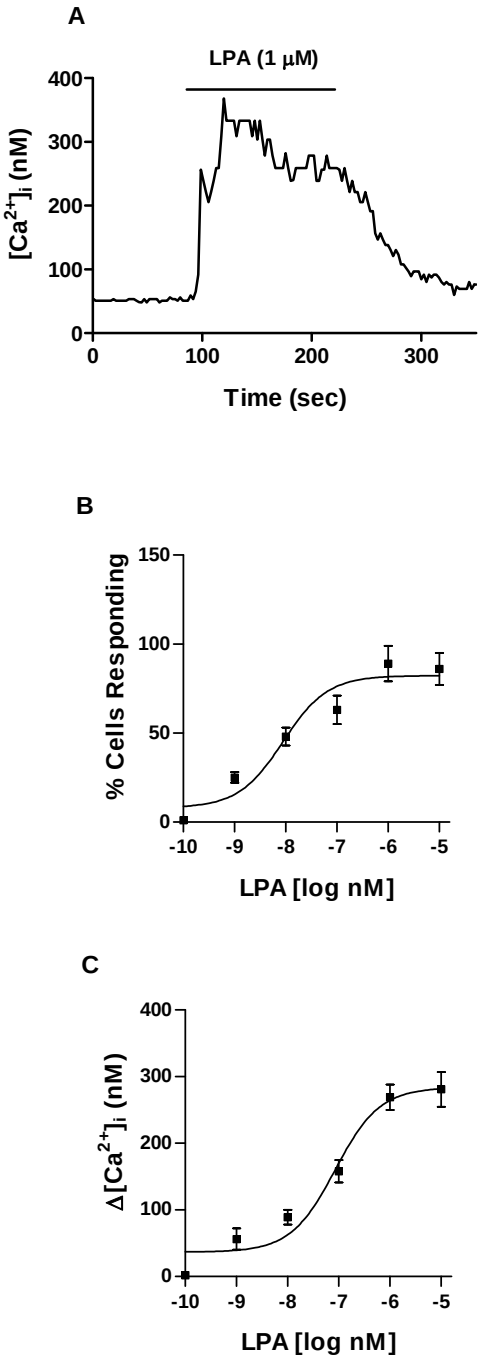
A
LPA (1 μM)
400
300
200
100
0
[Ca2+]i (nM)
0
100
200
300
Time (sec)
B
150
100
50
0
% Cells Responding
-10
-9
-8
-7
-6
-5
LPA [log nM]
C
400
300
200
100
0
Δ[Ca2+]i (nM)
-10
-9
-8
-7
-6
-5
LPA [log nM]

FIGURE 3.1

Figure 3.2. Desensitization following repetitive LPA exposure. Repetitive superfusion of enteric glia with LPA (1μM), 60s) in calcium-containing buffer, separated by a washout period of 200 s with buffer perfusion, evoked decreasing peak $[Ca^{2+}]_i$ responses (A). Subsequent treatment with ATP (100μM) generates a typically large Ca^{2+} signal, indicating diminished responsiveness to LPA is not attributable to internal Ca^{2+} store depletion. This tracing is typical of 124 glial responses. B. Compiled data from all generated Ca^{2+} signals. Data represent mean±SEM. $*P < 0.05$ versus initial response (Peak I).

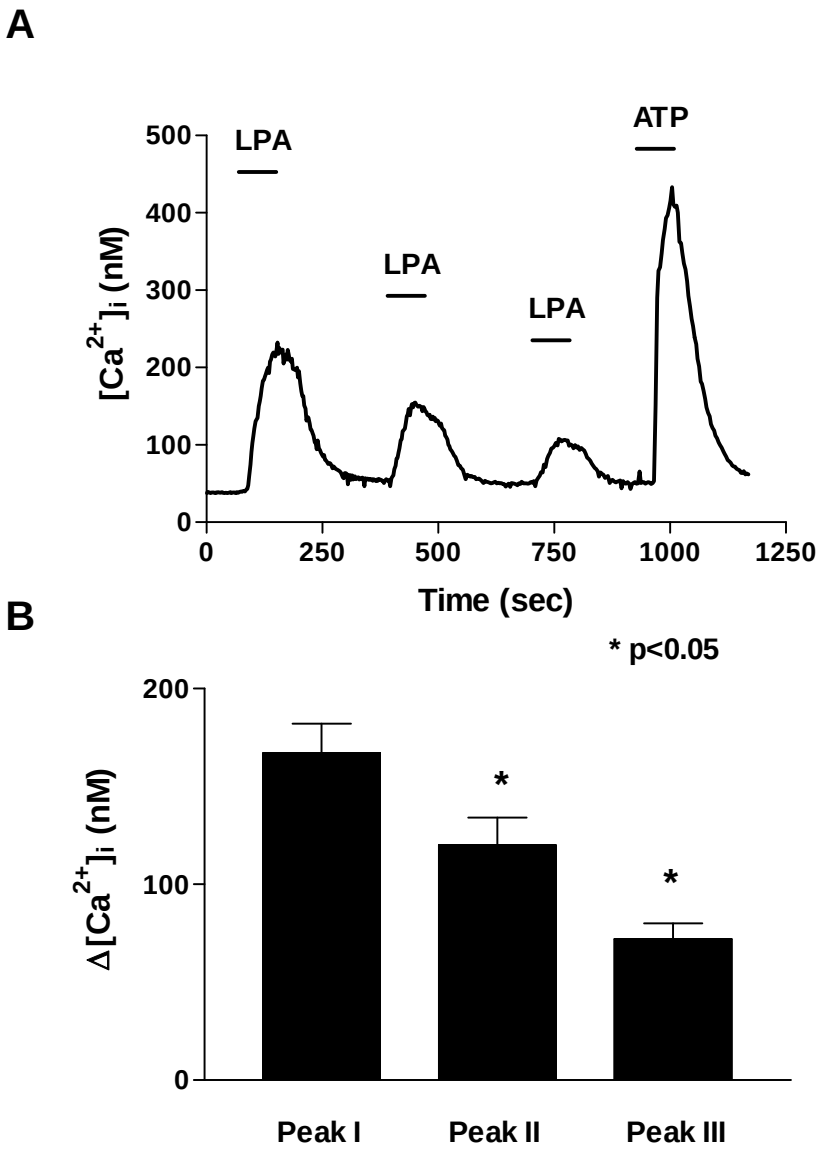
A
LPA
LPA
LPA
ATP
500
400
300
200
100
0
[Ca2+]i (nM)
0
250
500
750
1000
1250
Time (sec)
B
* p<0.05
200
100
0
Δ[Ca2+]i (nM)
*
*
Peak I
Peak II
Peak III

FIGURE 3.2

Figure 3.3. LPA signaling is PTX-sensitive. Cultured enteric glial cells were incubated with 100 ng/ml PTX for 24 h and then exposed to 1 μM LPA. Percentage of cells responding (A) and $\Delta[Ca^{2+}]_i$ (B) were measured. Data represent mean±SEM. **P* < 0.05 versus control (n=172 and 138 for control and experimental groups, respectively).

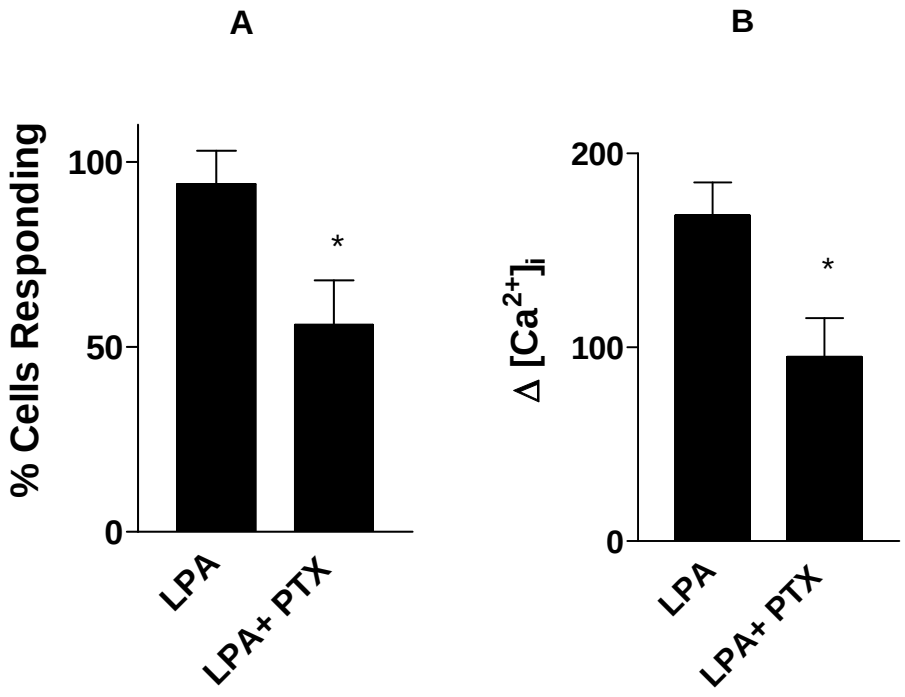
A
B
% Cells Responding
100
50
0
*
LPA
LPA+ PTX
Δ [Ca2+]i
200
100
0
*
LPA
LPA+ PTX

FIGURE 3.3

Figure 3.4. LPA signals involve intracellular and extracellular Ca^{2+}. A. Removal of extracellular Ca^{2+} from the perfusion buffer had no effect on initial peak responses to LPA (1μM, 120) but abolished the sustained plateau found in control conditions. At the end of experiment, perfusion with 100μM ATP induced $[Ca^{2+}]_i$ response, demonstrating cellular viability. (n=158). B. Depletion of intracellular Ca^{2+} stores by thapsigargin (1μM) blocks the subsequent response to LPA (1μM). (n=113)

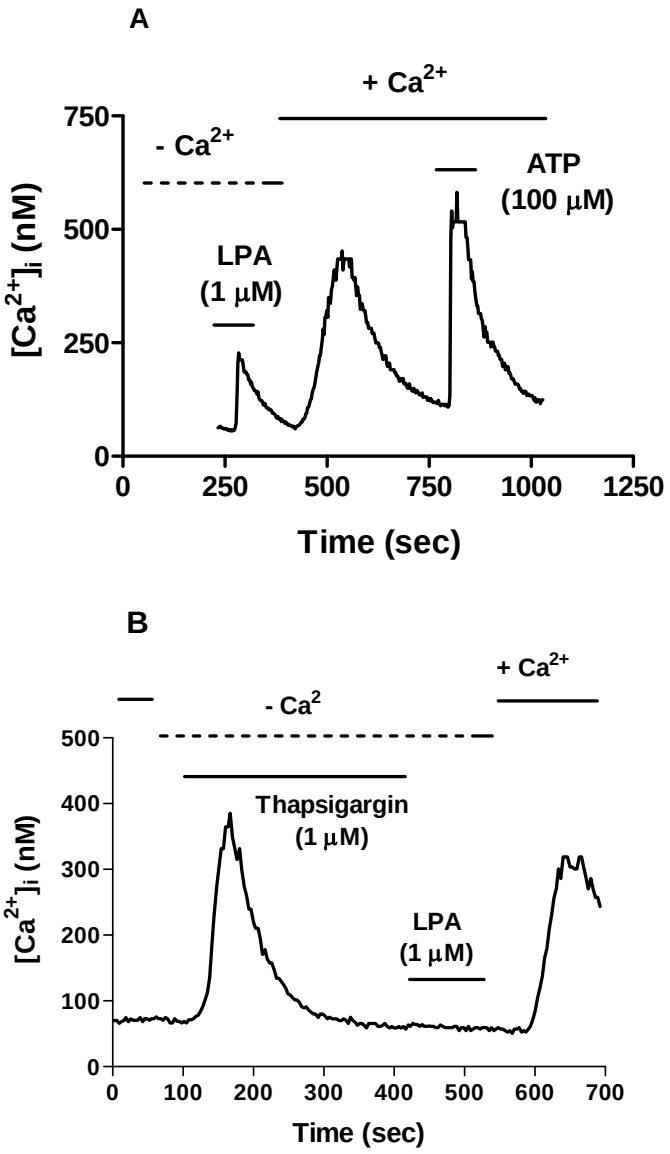
A
+ Ca2+
- Ca2+
ATP
(100 μM)
LPA
(1 μM)
[Ca2+]i (nM)
750
500
250
0
0
250
500
750
1000
1250
Time (sec)
B
+ Ca2+
- Ca2
Thapsigargin
(1 μM)
LPA
(1 μM)
[Ca2+]i (nM)
500
400
300
200
100
0
0
100
200
300
400
500
600
700
Time (sec)

FIGURE 3.4

Figure 3.5. LPA-induced Ca^{2+}_i mobilization involves the inositol 1,4,5-trisphosphate receptor. LPA was superfused for 120 s following pre-treatment with 2-aminoethoxydiphenylborate (2APB), an IP3R antagonist (n=141). Subsequent exposure to 100μM ATP evoked $[Ca^{2+}]_i$ response, demonstrating cellular viability.

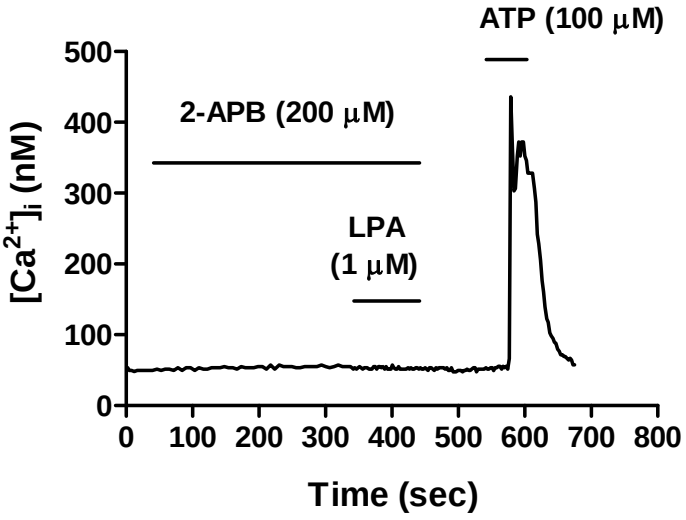
ATP (100 μM)
2-APB (200 μM)
LPA
(1 μM)
[Ca2+]i (nM)
500
400
300
200
100
0
0 100 200 300 400 500 600 700 800
Time (sec)

FIGURE 3.5

Figure 3.6. LPA-responsive G protein-coupled endothelial differentiation gene (EDG) receptor mRNAs EDG-2 and EDG-7 are expressed in the enteric nervous system. RT-PCR products of RNA extracted from 5 d old myenteric plexus primary cell cultures corresponding to EDG receptor coding sequences are shown in lanes 2 and 3: EDG-2 (925 bp) and EDG-7 (634 bp). Lanes 1 and 5 correspond to molecular size markers. Lane 4 represents the negative control (RT-PCR performed without the extracted RNA).

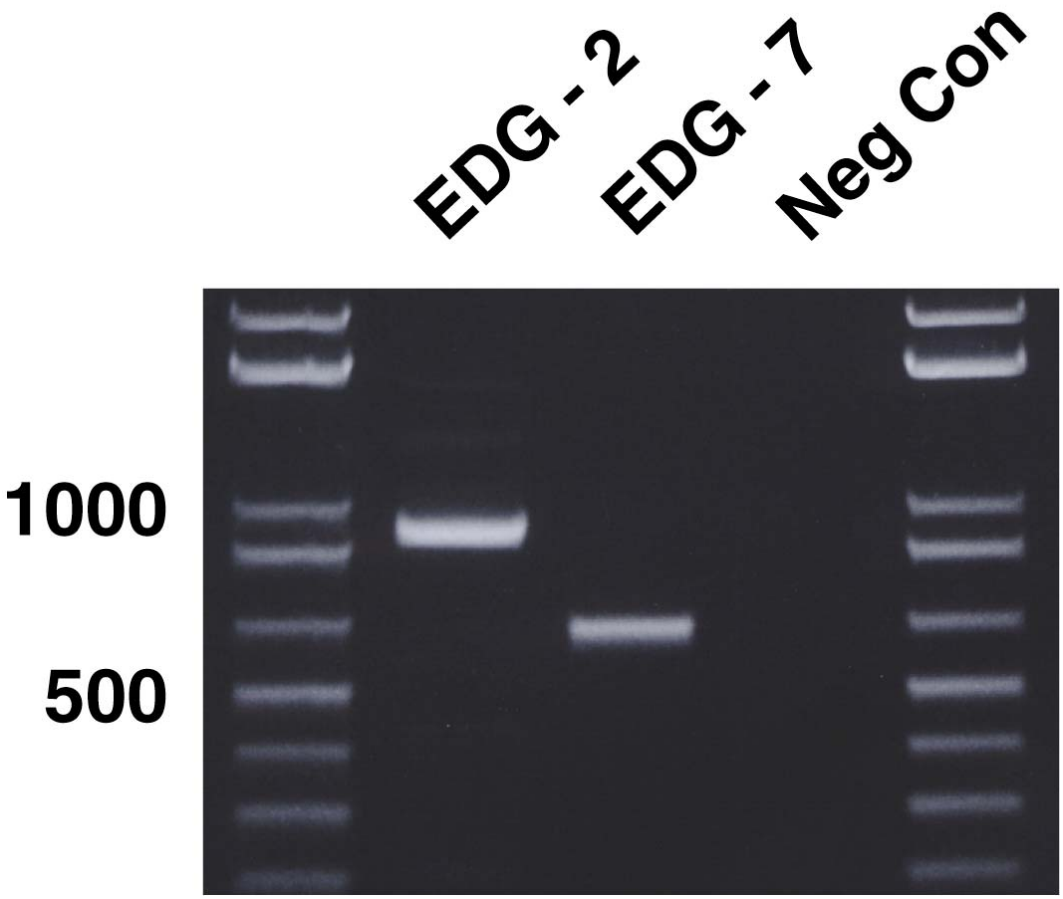

FIGURE 3.6

Figure 3.7. Immunocytochemical staining for EDG-2 and EDG-4. Myenteric plexus cultures from neonatal guinea pigs were stained for EDG-2 (A) or EDG-4 (B). Relevant negative controls are represented in panels C and D. Myenteric neurons were unstained.

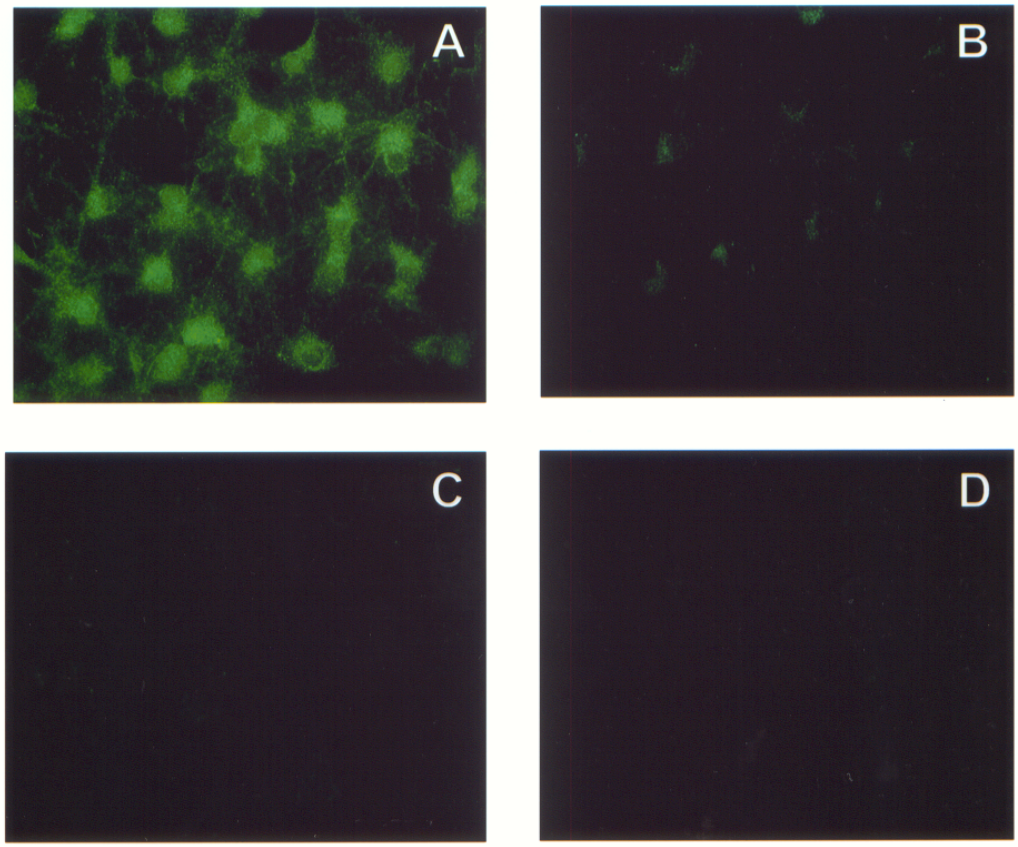

FIGURE 3.7

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CHAPTER 4

SPHINGOSINE-1-PHOSPHATE AND RELATED BIOACTIVE LIPIDS INDUCE EARLY RESPONSE GENE EXPRESSION IN C6 GLIOMA CELLS

[Note: This chapter has been slightly modified from a submitted manuscript: Segura, B.J., Zhang, W., Xiao, L., Logsdon, C., & Mulholland, M.W. 2005. Sphingosine-1-phosphate induces early response gene expression in C6 glioma cells. *Brain Res. Mol. Brain Res*. 133(2): 325-8.]

Abstract

Sphingosine-1-phosphate (S1P), a product of sphingomyelin catabolism, causes receptor-mediated calcium signaling in a variety of glial subtypes. Activation of early response gene expression by S1P and related lipids was examined in C6 glioma cells. S1P caused dose-dependent increases in *c-fos* mRNA at doses ranging from 1 μM (194±13% of control) to 100 μM (446±41%). Expression of *c-fos* was evident at 15 min (301±45%), greatest at 30 min (303±24%), and subsided within 1h (131±13%). Similar effects were found with the early response genes *jun-B.* Related lipids also induced *c-fos* expression. Sphingosine (10 μM—183±20%), lysophosphatidic acid (LPA, 100 μM—

188±41%), and sphingosylphosphorylcholine (SPC, 100 μM—420±44%) elicited significant rises in *c-fos* levels following 30 min of exposure. Pretreatment with PTX (100 ng/ml X 24h), reduced *c-fos* activation by S1P (100μM—187±6% vs. 411±27%) and LPA (100 μM—90±34% vs. 188±41%), but not by SPC (100μM—390±47% vs. 420±44%). RT-PCR analysis and sequencing demonstrated the presence of previously unidentified LPA-responsive EDG receptor mRNAs in C6 cells: EDG-2 and EDG-4.

Introduction

Sphingolipid metabolites have received increasing attention as a new class of signaling molecules. Sphingosine, sphingosine-1-phosphate (S1P), and lysophosphatidic acid (LPA) are members of this group of lipid signaling molecules. S1P is generated through sphingomyelin catabolism and has been implicated in an array of cellular events ranging from cellular proliferation, to shape change and cellular motility (1-3). S1P is functionally distinctive, serving either an extracellular or an intracellular role, depending upon cell type (1-4). The actions of S1P as an extracellular signaling molecule have been confirmed by the identification of a family of G-protein coupled receptors with affinity for S1P (Endothelial Differentiation Gene *[EDG]* receptors) (5-7). Although a number of recent reports support a role for S1P functioning via an extracellular receptor, the intracellular signaling pathways subsequently activated remain incompletely defined.

The current study was designed to investigate whether exposure of C6 glioma cells to S1P stimulates the expression of the immediate early response genes *c-fos* and *jun*-B. The *c-fos* proto-oncogene was among the first immediate early response genes identified and is well characterized. The product of the *c-fos* gene combines with the products of the *jun* family of proto-oncogenes to form heterodimeric transcription factor complexes. *Fos/Jun* heterodimers bind with high affinity to genes that contain the AP-1 DNA consensus sequence. When a *c-fos/c-jun* heterodimer binds to an AP-1 site it activates transcription, whereas *c-fos/jun-B* heterodimer binding at an AP-1 site represses transcription of the involved gene.

The current studies demonstrate that: 1) S1P mediates time- and dose-dependent expression of *c-fos* and *jun-B* expression; 2) c-*fos* expression is blocked by pertussis toxin

(PTX), a G_i antagonist, and by U73122, an inhibitor of phospholipase C; 3) the related sphingolipids sphingosylphosphorylcholine and LPA also stimulate *c-fos* expression and display differential sensitivity to PTX; and 4) EDG-2 and EDG-4 receptor mRNA is expressed in C6 cells.

Experimental Methods

Materials and Reagents

Sphingosine-1-phosphate (S1P), D-erythro-sphingosine (DSPH), lysophosphatidic acid (LPA), sphingosylphosphorylcholine (SPC), sphingomyelin (SM), C2-ceramide (C2-CER), sphingomyelinase (SMase), collagenase type V, soybean trypsin inhibitor (type I-S), penicillin-streptomycin solution, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were from Sigma Chemical (St. Louis, MO). U73122, and U73343 were obtained from Research Biochemicals International (Natick, MA). (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal calf serum, L-glutamate, and Trizol total RNA isolation kit were purchased from GIBCO Life Technologies, Inc (Rockville, MD). Radiolabeled (α-^{32}P)dCTP (3,000Ci/mmol), Rediprime DNA labelling kit were purchased from Amersham (Arlington, IL). Maximum Nytran nylon membranes and PDFV membranes were obtained from Schleicher and Schuell (Keene, NH). QuikHybe hybridization buffer was from Stratagene (La Jolla, CA). Qiaex II agarose gel extraction kits and Plasmid Maxi-Pre kits were obtained from Qiagen (Santa Clarita, CA). Midi select G-50 Sephadex spin columns were purchased from 5 Prime-3 Prime (Boulder, CO). Pre-cast

gels for SDS-polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-RAD (Hercules,CA). BCA protein assay reagents were purchased from Pierce (Rockford, IL).

cDNA Probes

cDNA probes used for these studies included *c-fos*, c-*jun*, *jun-B* and *jun-D* cDNA purchased from ATCC (American Type Culture Collection) and a cDNA for chicken gluceraldehyde-3- phosphate dehydrogenase (*GAPDH*) (Dugaiczyk et al, 1983). Plasmids containing probes were isolated from bacterial cultures using Qiagen Maxi-Pre isolation kits. A 920 bp Pst I fragment of *c-fos* and a 1 kb Pst I fragment of *cGAPDH* were cut with restriction enzymes and isolated from agarose gels using Qiaex II gel isolation kits. Probes for *jun-B, c-jun*, and *jun-D* were similarly prepared according to manufacturer instructions**.**

Cell Culture

C6 cells, a rat glioma cell line, were obtained from ATCC and cultured in DMEM media supplemented with 5% fetal calf serum, 100 IU/ml penicillin, and 100mg/ml streptomycin. Cells were used between passage 40 and 60. For experiments, cells were seeded at a $3X10^6$ cells/10 cm dish and grown in 5% CO_2 humidified condition. Cells at 80% confluence were depleted of serum overnight and then treated with sphingolipid compounds. When used, inhibitors were present during a preincubation period of 30 minutes and also during stimulation.

Northern Blot Analysis

Cells were harvested and total RNA isolated using Trizol reagent. Equal amounts of RNA (25μg/lane) were denatured and electrophoresed on 1.2% agarose-6% formaldehyde gels, transferred to nylon membranes, and immobilized by UV cross-linking (Stratalinker, Stratagene). cDNA probes were labeled with ^{32}P dCTP using a random hexanucleotide priming kit followed by purification using a G50 sephadex spin column. Membranes were pre-hybridized for 20-30 minutes at 68 °C in QuikHybe suspension supplemented with 100μg/ml sonicated salmon sperm DNA. Hybridization with the ^{32}P-labeled probes was carried out at 68 °C for 1 hour. Membranes were washed in 2X SSC/0.1%SDS at room temperature, followed by 0.1XSSC/0.1%SDS at 60 °C, and exposed to X-ray film (Kodak X-Omat AR, Rochester,NY) with intensifying screens at -70°C. GAPDH marker was used to normalize signals. All experiments were repeated at least three times.

RNA extraction and Reverse Transcription (RT)

Total cellular RNA was isolated from C6 cell cultures using TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to manufacturer's directions. Single strand cDNA synthesis was performed as follows: 20 µl of reverse transcription mixture contained 1 µg of DNase I pre-treated total RNA, 0.5 µg of oligo d(T) primer (Roche Molecular Biochemicals, Indianapolis, IN), 4 µl of 5 x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides (Roche), 40 units of RNase inhibitor (Promega, Madison, WI),

and 200 units of reverse transcriptase (SuperScript II RT, Life Technologies). The RT reaction was carried out at 42^{o}C for 50 min followed by heat inactivation at 70^{o}C for 15 min.

Polymerase Chain Reaction (PCR)

50 µL of PCR reaction mixture contained 1 µl of RT products, 1X cDNA PCR reaction buffer (Clontech Laboratories, Inc., Palo Alto, CA), 400 nM of each primer, 200 µM of dNTP mix, and 1X Advantage cDNA polymerase mix. PCR was carried out using a PTC-200 DNA engine (MJ Research, Inc., Waltam, MA). Samples were denatured initially at 94^{o}C for 1 min and the PCR was performed as follows: 32 cycles of 30 sec at 94^{o}C, 30 sec at 65^{o}C and 1 min at 72^{o}C for EDG-2; 42 cycles of 30 sec at 94^{o}C, 30 sec at 65^{o}C and 1 min 30 sec at 72^{o}C for EDG-4, followed by the final extension at 72^{o}C for 7 min.

PCR primers used for rat C6 cell mRNA detection were deduced from human, mouse and rat published sequences. The nucleotide sequences of sense and antisense primers with the expected product size are as follows: EDG-2, AGT ATC TAG CCA CAG AAT GGA ACA CT (sense) and AAC ACA CAT CGA GTA GCA AGA C (antisense) (735 bp product); EDG-4, CAT GGG CCA GTG CTA CTA CAA CGA G (sense) and CGG AAG GTG CGG CGC ATC TCA G (antisense) (912 bp product). For negative controls, PCR reactions were performed for each of the primer pairs in the absence of transcript as others have reported.

Sequencing and Data Analysis

PCR products were electrophoretically analyzed on 1.2% agarose gel containing ethidium bromide. DNA bands were excised from the gel and purified as follows: gel slices were crushed in the tube and sample DNA was extracted twice with buffered phenol and once with phenol:chloroform:isoamyl (25:24:1) followed by ethanol precipitation with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol and washing with 70% ethanol. The purified DNA fragments were directly sequenced by the DNA sequencing core at the University of Michigan using Applied Biosystems DNA sequencers.

Data Presentation

Results are expressed as mean $\pm$ SEM. Data were analyzed by analysis of variance, with significance was accepted as $P < 0.05$.

Experimental Results

S1P dose-dependently increased c-*fos* mRNA expression in C6 cells over a range of 10 nM to 100 μM [Figure 1]. These concentrations were selected based on preliminary studies using fura-2-based increments in intracellular Ca^{2+} as a marker of cellular responsiveness. C6 cells reversibly responded to S1P (10 nM to 100 μM) with increases in intracellular calcium concentration. Subsequent exposure to ATP (100 μM) also induced an increment of intracellular calcium concentration, indicating continued

viability of C6 cells exposed to S1P. To evaluate the time course of c-*fos* transcription, C6 cells were treated with S1P (10 μM) for 15, 30, 60, or 120 minutes. Expression of the *c-fos* transcript was significantly increased by 15 minutes, maximal at 30 minutes, and returned to basal expression by 1 hour [Figure 2]. Expression of *jun*-B mRNA was also significantly increased at 15 minutes and maximal at 30 minutes, but remained elevated relative to baseline for up to 2 hours [Figure 3]. Expression of *jun*-B mRNA was stimulated in a dose-dependent fashion after incubating C6 cells for 30 minutes with S1P over a concentration range from 10 nM to 100 μM [Figure 4].

Related lipids also induced *c-fos* expression. Sphingosine (10 μM—183±20%), lysophosphatidic acid (LPA, 100 μM—188±41%), and sphingosylphosphorylcholine (SPC, 100 μM—420±44%) elicited significant rises in *c-fos* levels following 30 min of exposure.

Subsequent experiments were conducted with S1P at 10 μM for 30 minutes. Inhibitors were added 30 minutes prior to agonist and were continued for the S1P incubation period. Co-incubation with the phospholipase C antagonist U73122 (10 μM) abolished *c-fos* expression stimulated by S1P [Figure 5]. Incubation with U73343 (10 μM), the inactive analogue, had no inhibitory effect on S1P-stimulated *c-fos* mRNA levels. Similar results were obtained with *jun*-B [Figure 6].

Pertussis toxin (100 ng/ml, 24 h) pre-exposure inhibited S1P-induced *c-fos* expression by 80% [Figure 7], and LPA-induced *c-fos* expression by 48% [Figure 8], whereas SPC-induced *c-fos* expression was not significantly affected [Figure 9].

RT-PCR analysis demonstrated that mRNAs for EDG receptors sensitive to LPA (EDG-2 and EDG-4) were present in C6 cells [Figure 10].

Discussion

The current studies demonstrate that sphingosine-1-phosphate and the related lipids lysophosphatidic acid and sphingosylphosphorylcholine stimulate early response gene expression in C6 cells and that S1P induces c-*fos* expression occurs through a receptor-dependent mechanism acting via G_i and phospholipase C. The data that support this conclusion are four-fold: 1) S1P stimulates time- and dose-dependent increases in c-*fos* and *jun*-B mRNA expression; 2) S1P effects on c-*fos* expression are suppressed by pertussis toxin, an inhibitor of G_i proteins; 3) S1P effects on c-*fos* expression are suppressed by U73122, an inhibitor of PLC; and 4) LPA receptors EDG-2 and EDG-4 are expressed in C6 cells.

While glial cells have traditionally been regarded as having passive, supportive functions in neural tissue, recent evidence suggests that these cells play an active role in information transfer within the brain, spinal cord, and at peripheral sites like the enteric nervous system (8, 9). The finding that S1P is a potent agonist for immediate early gene expression signaling in C6 glioma cells, and that related lipids also have effects in these cells, implies that there may be multiple mechanisms for lipid signaling in glia.

Sphingolipid metabolites such as S1P and its receptors have recently been shown to affect a wide variety of biological processes including cellular proliferation, migration and development, neurite retraction, and neuronal survival and differentiation (1-4, 10). S1P causes Ca^{2+} signaling in cultured CNS astrocytes and Ca^{2+} transients and shape changes in C6 glioma cells (11). This study provides the first evidence for sphingolipid-

induced early response gene expression in C6 cells. The results presented here are congruent with previously described effects of S1P and related lipids upon Ca^{2+} signaling in glia.

Goodemote *et al*. initially observed that S1P effects in Swiss 3T3 fibroblasts were pertussis toxin sensitive, suggesting this lipid possessed activity that resembled other agonists of G-protein-coupled receptors (GPCRs) (11). The recent characterization of a family of GPCRs (EDG receptors) with affinity for S1P and related lipids has established a role for receptor-mediated lipid signaling events (10). Previously an orphan receptor, EDG-1 was the first putative S1P receptor to be identified (12). To date, seven additional EDG receptors have been identified that are differentially activated by S1P, SPC and LPA (5-7).

The variety of sphingolipid signaling molecules that were demonstrated to induce c-*fos* expression in C6 cells implies that multiple EDG receptors may be present in glia. EDG-1, EDG-3, EDG-5/H218/AGR16, EDG-6, and EDG-8 are responsive to S1P and, to a lesser degree, SPC, whereas EDG-2/*Vzg*-1, EDG-4, and EDG-7 appear to be selectively sensitive to LPA (5-7, 13). Sato *et al*. have previously shown, by Northern blot analysis, that C6 glioma cells express mRNA for EDG-1 and EDG-5 (14). The present study demonstrated that C6 cells also express mRNA for LPA receptors. Using RT-PCR, EDG-2 and EDG-4 receptor mRNAs were demonstrated in cultured C6 cells. The rat C6 cell EDG-2 and EDG-4 receptors exhibited strong sequence homology with known mouse, rat and human EDG-2 and EDG 4 receptors, respectively. The presence of a pair of LPA-activated EDG receptors is consistent with the production of Ca^{2+} responses in C6 cells after stimulation with LPA as previously shown by Hildenbrandt and

Hildebrandt (15) and Tas and Koschel (16) and c-*fos* expression induction by LPA in C6 cells as we present in this report. The mRNA transcript for another LPA-specific receptor, EDG-7, was not detected in C6 cells.

EDG-1, EDG-3, and EDG-5 have all been demonstrated to couple to G_i, but EDG-3 and EDG-5 are also capable of interacting with G_q in transfected Sf9 and HEK293 cells (6). If rat EDG-5 couples to G_q in C6 cells, S1P-induced c-fos expression may only be partially sensitive to PTX exposure. In many cell types, G_q and G_i proteins link GPCRs with PLC. The current studies convincingly demonstrate that S1P-induced c-*fos* expression is mediated by PLC as this action is antagonized by the PLC inhibitor U73122, but not by the inactive analog, U73343. Other investigators have also reported that S1P signaling involves PLC (5).

In support of a physiological source of S1P, recent studies indicate that upon activation with prothrombotic stimuli, platelets release amounts of S1P that increase serum levels to 500 nM, similar to concentrations used in these studies (4). The recruitment and activation of platelets during an inflammatory response within nervous tisssue could produce local concentrations of S1P that would affect glia.

In conclusion, C6 glioma cells exhibit receptor-mediated early response gene induction following treatment with sphingosine-1-phosphate. The results suggest that C6 cells respond to bioactive lipids via a receptor-mediated mechanism that involves phospholipase C, leading to acute transcriptional events.

Acknowledgments

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Figures

Figure 4.1. Northern blot analysis of dose-dependent expression of *c-fos* mRNA upon exposure to S1P. C6 cells were treated with S1P over a concentration range of 0.01 to 100 μM. Blot is representative of data from four different experiments. After hybridization, membranes were rehybridized using a *GAPDH* probe to control for variations in gel loading and transfer efficiency. Data are compiled from an average of four separate experiments. Concentrations of 1, 10, and 100 μM were significantly different from basal.

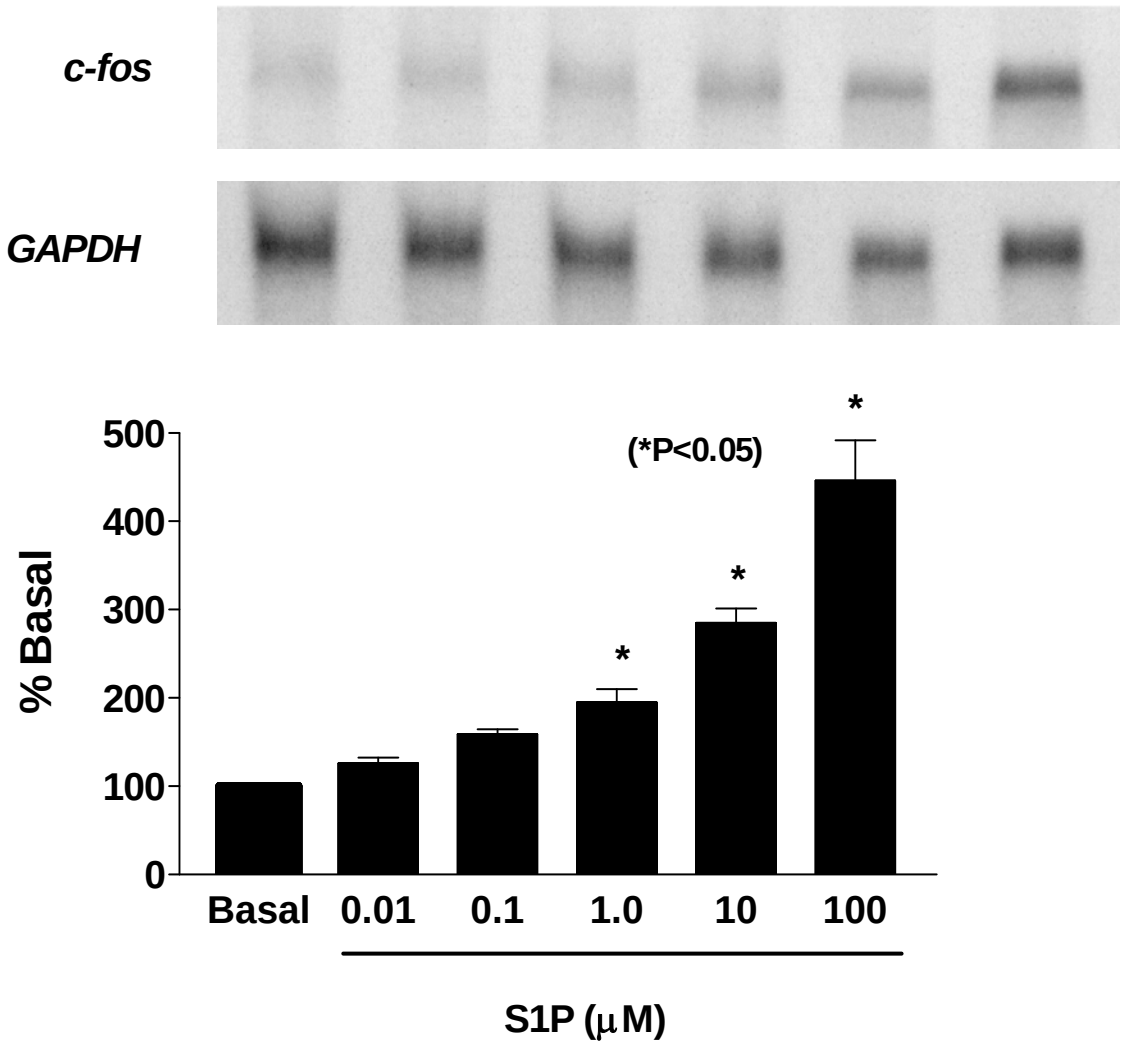
c-fos
GAPDH
(*P<0.05)
*
*
*
% Basal
500
400
300
200
100
0
Basal
0.01
0.1
1.0
10
100
S1P (μM)

FIGURE 4.1

Figure 4.2. Time-dependent expression of *c-fos* mRNA. C6 cells were treated with S1P (10 mM) for 15, 30, 60 and 120 min. Significant increases in *c-fos* were noted by 15 min. Blot is representative of data from four experiments.

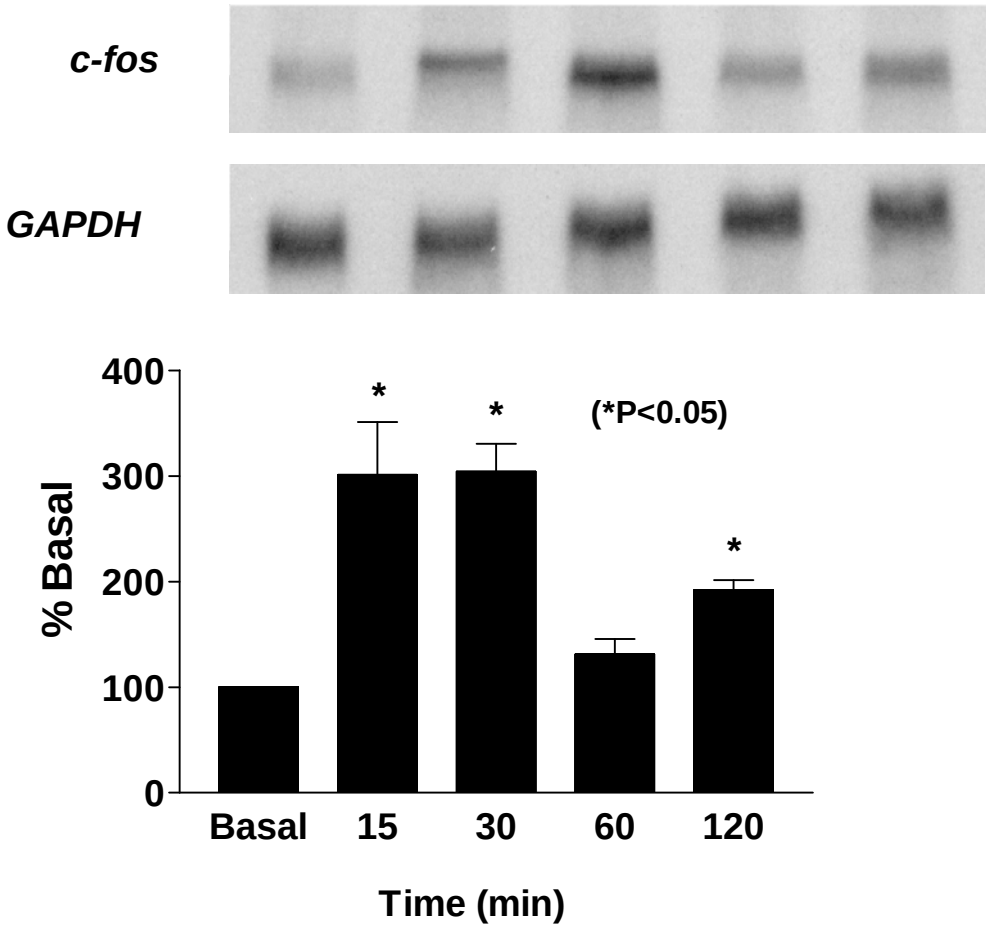
c-fos
GAPDH
400
300
200
100
0
% Basal
*
*
*
(*P<0.05)
Basal
15
30
60
120
Time (min)

FIGURE 4.2

Figure 4.3. Northern blot analysis of dose-dependent expression of *jun*-B mRNA upon exposure to S1P. C6 cells were treated with S1P over a concentration range of 0.01 to 100 μM. Data are compiled from an average of four separate experiments. Concentrations of 10 and 100 μM were significantly different from basal.

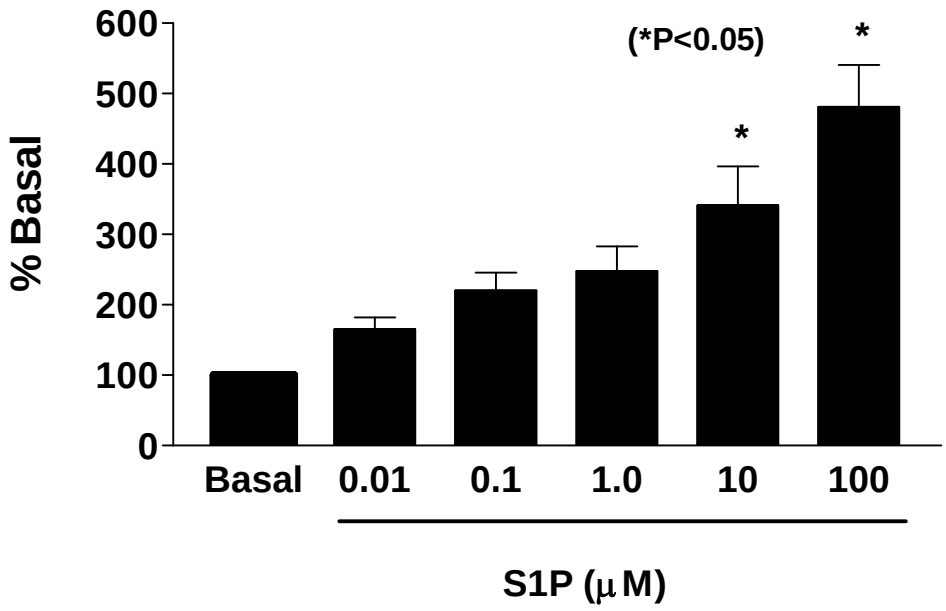
600
500
400
300
200
100
0
% Basal
(*P<0.05)
*
*
Basal
0.01
0.1
1.0
10
100
S1P (μM)

FIGURE 4.3

Figure 4.4. Time-dependent expression of *jun-B* mRNA. Significant increases in *jun*-B were noted by 15 min. C6 cells were treated with S1P (10 mM) for 15, 30, 60 and 120 min.

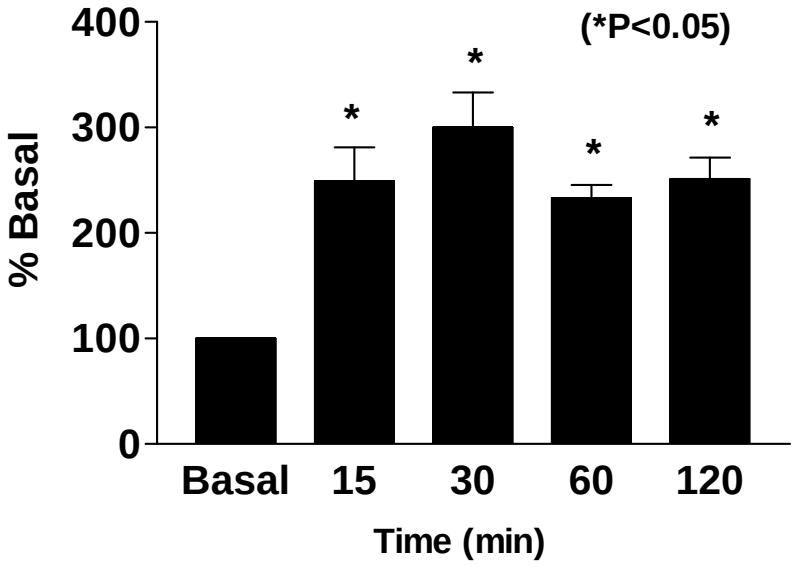

FIGURE 4.4

Figure 4.5. S1P effects upon *c-fos* involve phospholipase C activation. C6 cells were exposed to S1P (10 μM) alone, or in addition to either U73122 (10 μM, a PLC antagonist) or U73343 (10 μM, an inactive PLC antagonist) for 30 min before *c-fos* determination.

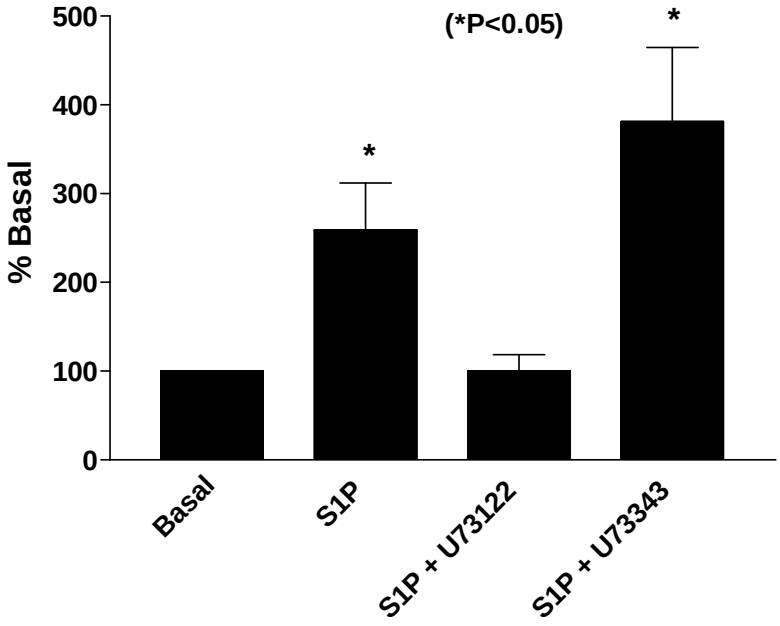
(*P<0.05)
*
*
% Basal
500
400
300
200
100
0
Basal
S1P
S1P + U73122
S1P + U73343

FIGURE 4.5

Figure 4.6. S1P effects upon *jun*-B involve phospholipas C activation. C6 cells were exposed to S1P (10 μM) alone, or in addition to either U73122 (10 μM, a PLC antagonist) or U73343 (10 μM, an inactive PLC antagonist) for 30 min before *jun*-B determination.

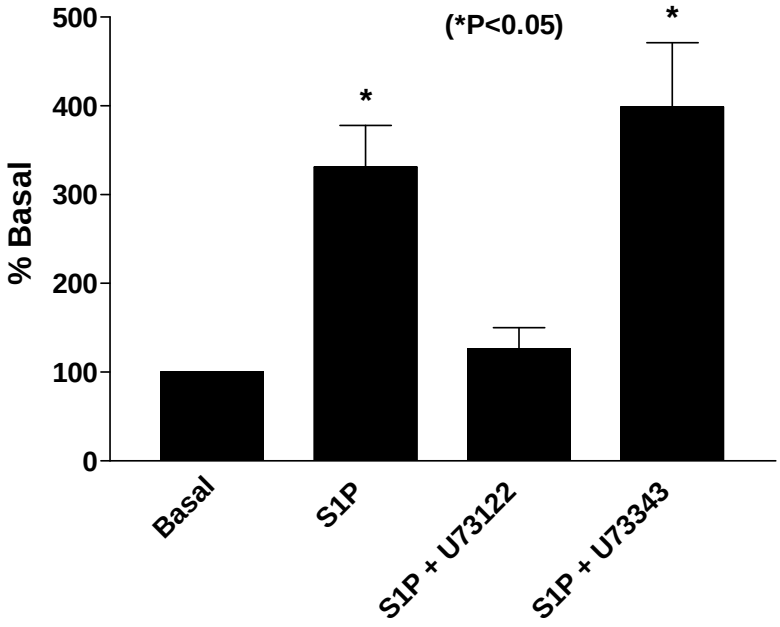
(*P<0.05)
% Basal
500
400
300
200
100
0
*
*
Basal
S1P
S1P + U73122
S1P + U73343

FIGURE 4.6

Figure 4.7. S1P effects are PTX-sensitive. Following incubation with pertussis toxin (100 ng/ml) for 24 hours, C6 cells were exposed to S1P (10, 100 μM).

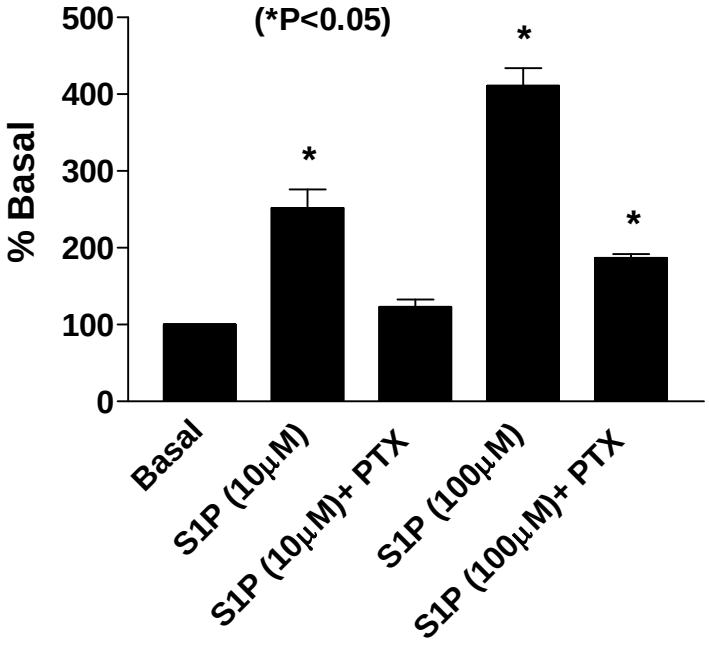
(*P<0.05)
% Basal
500
400
300
200
100
0
*
*
*
Basal
S1P (10μM)
S1P (10μM)+ PTX
S1P (100μM)
S1P (100μM)+ PTX

FIGURE 4.7

Figure 4.8. LPA effects are PTX-sensitive. Following incubation with pertussis toxin (100 ng/ml) for 24 hours, C6 cells were exposed to LPA (10, 100 μM).

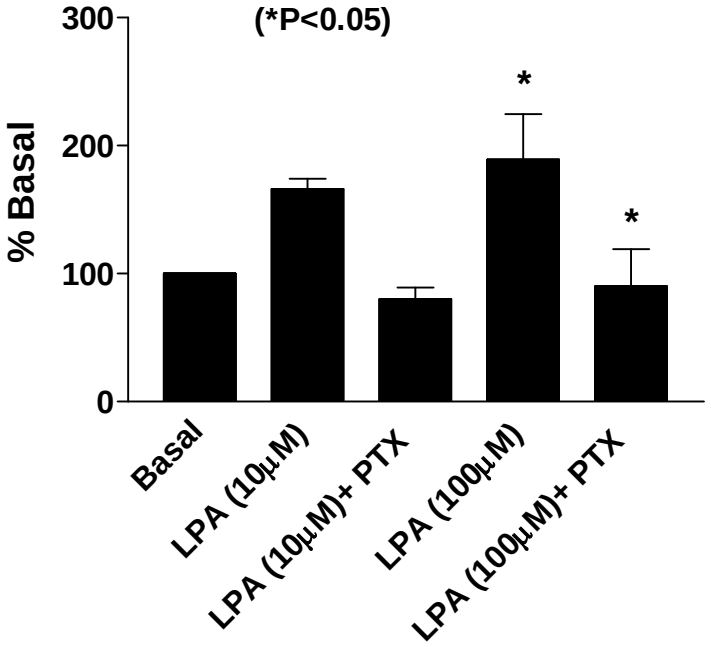
(*P<0.05)
% Basal
300
200
100
0
*
*
Basal
LPA (10μM)
LPA (10μM)+ PTX
LPA (100μM)
LPA (100μM)+ PTX

FIGURE 4.8

Figure 4.9. SPC effects are PTX-insensitive. Following incubation with pertussis toxin (100 ng/ml) for 24 hours, C6 cells were exposed to S1P (10, 100 μM).

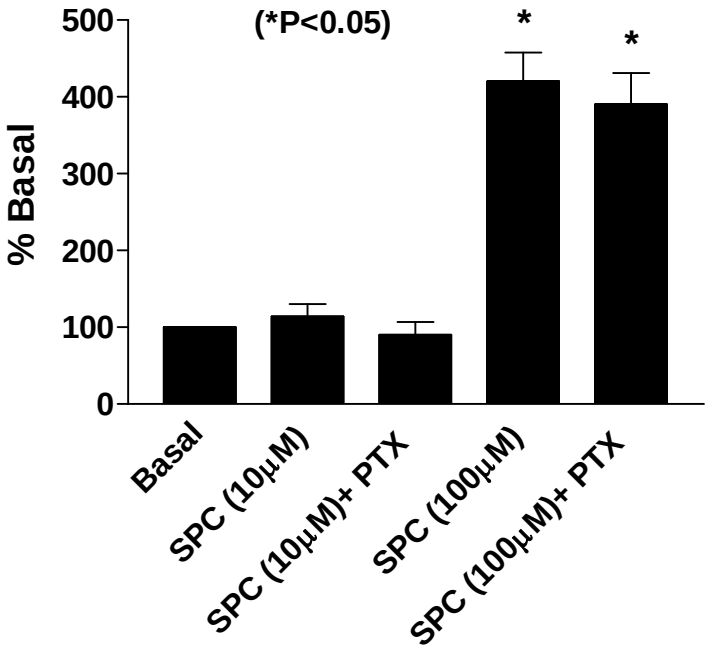
(*P<0.05)
*
*
500
400
300
200
100
0
% Basal
Basal
SPC (10μM)
SPC (10μM)+ PTX
SPC (100μM)
SPC (100μM)+ PTX

FIGURE 4.9

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CHAPTER 5

CONCLUSION

Summary

The work presented in this thesis was aimed at understanding the mechanisms of how signaling occurs in enteroglia. This dissertation describes the effects of bioactive lipids on glial cells of the gastrointestinal tract, testing the overarching hypothesis that sphingosine-1-phosphate and related compounds affect signaling in these cells. More specifically, these lipids are shown to activate calcium signaling in enteric glia, where numerous lipid-specific receptors are identified. Efforts focus on elucidating the mechanisms of the signaling pathways involved. Further, members of this diverse class of lipids are found to induce early response gene expression in C6 glioma cells, which are known to both respond to bioactive lipids with calcium signaling and express various receptors that transduce bioactive lipid effects.

The bioactive lipids sphingosine-1-phosphate (S1P), lysophosphatidylcholine (LPA) and sphingosylphosphorylcholine (SPC) have been described in the scientific literature as unique signaling molecules in that they, while lipids, activate G protein-coupled receptors (Endothelial Differentiation Gene receptors) on cell surfaces (1-11). Recently, lysophosphatidylcholine (LPC) has joined these ranks as it, too, has been found to serve as a ligand for yet another G protein-coupled receptor (which, incidentally, also share analogous activation by SPC) (11-13). That such a diverse and rapidly expanding array of receptors may be selectively activated by these compounds emphasizes cells, including glia, have complex signaling mechanisms which go far beyond the original

notion that only peptides are responsible for transducing receptor-mediated responses. Even more intriguing is the consideration that at least two of these lipids (S1P and SPC) are believed to function through dual mechanisms: extracellularly via their respective G protein-coupled receptors and intracellularly via one or more undefined receptors which appear to be analagous to the inositol trisphosphate receptor in that they lead to the mobilization of intracellular calcium stores (1-11). Adding to the level of complexity, it has been shown that a variety of signaling molecules, including the LPA and S1P, stimulate intracellular S1P production (1-11). In all, given the breadth of their effects upon cells from cytoprotection to proliferation (1-13), further uncovering the mechanisms for each of these lipids and determining their physiological, and potentially pathological, roles promises to continue to be an intriguing area of research.

It is an exciting time for glial biologists. After decades of being overshadowed by neurons, it is clear that glia also serve critical roles in neural systems (14). If, in spite of substantial progress, much work remains at the level of the central nervous system, then far greater effort is due in the enteric nervous system (ENS) where glia are still a great mystery. Given the recent advances within the gastrointestinal tract to delineate the contribution of glia in health and disease, these once anonymous cells are now recognized as active elements in the gut (15-20). The work presented here strongly supports a potential role for glia in information transfer within the walls of the bowel and beyond as we demonstrate the increasing diversity of signaling events displayed by these cells.

Chapter 1 of this thesis lays the foundation of this investigation by demonstrating that enteric glia display similarities to glia of the central nervous system both structurally and functionally. Upon review of the literature, including evidence from our laboratory,

one readily appreciates the potential for glial involvement in active signaling within the gut (15-24). Data are provided depicting responsiveness to the neuroligands ATP and endothelin with calcium signaling and these mechanisms are dissected. Calcium signaling is a foundational element of intra- and intercellular communication. This is seen in the central nervous system where glial cells are known to propagate signals among themselves and also felt to have the potential to modulate neuronal activity and synaptic transmission (14).

Although somewhat teliologic in argument, these results beg the question, "Why would cells be equipped to signal in this analogous manner if not involved in the essential transfer of information in the gastrointestinal tract?" The sentinel observation of enteroglial agonist-induced calcium by earlier studies in our laboratory raised the possibility that these cells serve as messengers under physiologic circumstances. Others have similarly proposed that plasticity is found within the ENS and that neurons and glia alike play a prominent role in this microenvironment through propagation of signals, cytoskeletal changes and neurotransmission (17).

Further, on the pathophysiologic front, by identifying molecules involved in inflammation or disease states which in turn induce glial signals, a potential link between the two is established. While definitive data linking the two are not made by this body of work, the postulate that enteric glia and inflammation and disease within the GI tract are associated represents a natural extension of this research. The observation by Bush, et al. that the ablation of enteroglial cells in transgenic mice leads to fatal fulminant jejuno-ileitis serves as further evidence that these cells may have an immunomodulatory role in the gastrointestinal tract (18-20). Other studies suggest that enteric glia may play a role

in Hirschsprung's disease, a congenital condition wherein the enteric nervous system fails to development normally. Aberrant endothelin signaling is a major culprit for this gastrointestinal defect remedied only by surgery in young infants, once again hinting at an association between enteric glia and its neuroligands (of note, enteric neurons are unresponsive to endothelin) (25-39). Chapter 1 serves as the introduction to this thesis by substantiating that enteric glia have unique signaling characteristics, without which enteric neurons, the gastrointestinal tract, and the organism as a whole would be incapable of survival.

In Chapter 2, a study of the actions of a novel lipid compound in the ENS is revealed. This series of investigations was conducted with again the long-term goal of understanding neurotransmission within the gut. Enteric glia, rather than enteric neurons, are found to selectively respond to a bioactive lipids. The sphingomyelin metabolite sphingosine-1-phosphate (S1P) causes dose-dependent, pertussis toxin-insensitive calcium (Ca^{2+}) signaling utilizing extracellular and intracellular Ca^{2+} in a manner which appears to be receptor-mediated. Several S1P-coupled endothelial differentiation gene (EDG) receptor mRNAs (EDG-1, EDG-3, and EDG-5) are found within the ENS with enteric glial cells demonstrating strong expression of both EDG-1 and EDG-3 and weak expression of EDG-5. Further, other sphingomyelin cycle components (sphingomyelin, sphingomyelinase, and sphingosine) also activate Ca^{2+} signaling in enteric glia, leading to the postulate that these cells are capable of processing sphingolipids to elicit intracellular S1P responses. The related lipids lysophosphatidic acid (LPA) and sphingosylphosphorylcholine (SPC)—agonists of differing EDG receptors—are also found to Ca^{2+} signaling in enteric glia. One conclusion from these data is that there are

multiple lipid-activated signaling mechanisms exist in these cells, leading to the hypothesis that S1P and other bioactive lipids function as novel signaling molecules within the ENS to facilitate information transfer.

The natural extension of the studies in Chapter 2, a more detailed investigation into the role of LPA in enteric glia, is the substance of Chapter 3. Again, enteroglia are found to selectively respond to this product of phosphatidylcholine metabolism while enteric neurons fail to generate calcium signals in response to this lipid. LPA causes dose-dependent calcium (Ca^{2+}) signaling in these cells at what are believed to be physiologic levels. Its acts through what appears to be an extracellular receptor, at least partially involving a G protein of the G_i subtype. RT-PCR analysis demonstrated the presence of two LPA-coupled EDG receptor mRNAs (EDG-2 and EDG-7) in myenteric plexus primary cultures and EDG-2 expression in glial cells of the ENS was confirmed immunocytochemically. Taken together with our previous data on S1P signaling in enteric glia, the conclusion was drawn from this body of evidence uncovering the role of LPA that multiple lipid-activated signaling mechanisms exist in these cells.

Although these data supported a role for S1P functioning via an extracellular receptor in glia, the intracellular signaling pathways subsequently activated remained incompletely defined. The studies in Chapter 4 were designed to disclose whether S1P and related lipids would induce expression of the immediate early response genes *c-fos* and *jun*-B in a model glial cell line: C6 glioma cells. These proto-oncogenes, members of a well-characterized family of immediate early response genes, were chosen with the hope of further characterizing lipid-mediated intracellular signaling events at the transcriptional level. The results demonstrated that: 1) S1P mediated time- and dose-

dependent expression of *c-fos* and *jun-B* expression; 2) c-*fos* expression was blocked by pertussis toxin (PTX), a G_i antagonist, and by U73122, an inhibitor of phospholipase C; 3) the related sphingolipids sphingosylphosphorylcholine and LPA also stimulated *c-fos* expression and displayed differential sensitivity to PTX; and 4) EDG-2 and EDG-4 receptor mRNA was expressed in C6 cells.

Future Directions

A conclusion of this thesis would be remiss without at least a brief discussion of ideas for future efforts. They likely include the identification and further characterization of other bioactive lipids affecting glial signaling (see following section for preliminary data on the related lipids sphingosylphosphorylcholine and lysophosphatidylcholine) and a deeper look into the biological effects of bioactive lipid treatment in the ENS. For example, results from our lab and others suggests that these lipid molecules may induce cytoskeletal changes and gene expression in glial cells, including those of the ENS. The evolution of this and other projects that both grew and will grow out of the initial observation one evening a few years ago that S1P strongly activates calcium signaling in enteroglia shall prove fascinating. Creativity and persistence will be essential to overcome limitations of the current model, including the possible generation of a reliable enteric glial cell line or the ability to otherwise produce large qualities of purified primary cell populations. Decoding the signals of glia as they actively participate in information transfer in the gastrointestinal tract will continue to generate excitement and attract more scientists to this rapidly growing field. I am eager to both witness and participate in this

timely movement as a developing scientist and clinician. In the ensuing paragraphs, some exciting preliminary data will be shared as an adjunct to data born by this thesis followed by a summary diagram depicting the data discussed in this final chapter [Figure 5.7].

A Role for Sphingosylphosphorylcholine (SPC) and Lysophosphatidylcholine (LPC) in the Enteric Nervous System.

Abstract

Bioactive lipids consist of a growing family of molecules with the ability to transduce a variety of cellular responses in numerous cell models. Sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC) are bioactive lipids that have been studied for years yet have only recently been shown to activate receptor-mediated pathways. In this section, the effects of SPC and LPC upon calcium signaling in glial cells of the enteric nervous system (ENS) are revealed. In short, SPC and LPC both cause robust calcium signaling in enteric glia. These responses appear to be receptor mediated as they rapidly desensitize with repetitive exposure. Further these two compounds have differential sensitivity to pertussis toxin (PTX): SPC responses are attenuated with PTX pretreatment (and, thus, are likely G_i coupled) whereas LPC responses are not. SPC and LPC represent two additional means by which enteric glia may participate in information transfer within the gut.

Introduction

As has been discussed in this thesis, our understanding of the role of glia in the gastrointestinal tract is evolving at a rapid pace. The evidence for these previously enigmatic cells to serve in an active fashion, like their counterparts in the central nervous system is overwhelming. That they possess the ability to undergo calcium signaling in response to a diverse array of compounds suggests they may be active participants in information transfer in the neural circuit found within the wall of the bowel. Other studies suggest that they may be clinically relevant as potential culprits in inflammatory bowel disease and Hirschsprung's disease.

Sphingosylphosphorylcholine (SPC) is a bioactive lipid that participates in a number of biological processes by serving as a signaling molecule (2, 3, 6, 10- 13). As discussed in previous chapters, early reports found that SPC shared activation of select Endothelial Differentiation Gene receptors with sphingosine-1-phosphate. The first G protein-coupled receptor found to be specific for SPC was OGR1 (40). Due to similarities found with Platelet Activating Factor (PAF) receptor, it was postulated that a subfamily of receptors existed for lysolipids possessing the phosphorylcholine moiety (12). Lysophoshatidylcholine (LPC) is such a naturally occurring compound that has been described to participate in a variety of cellular effects including inflammation and atherosclerosis (11). Indeed, LPC was recently identified as the ligand for the immunoregulatory receptor G2A (41, 42). Further, emphasizing the ability of the related compounds to function similarly, both SPC and LPC were subsequently discovered to serve as dual ligands for the G protein-coupled receptor GPR4 (12).

Sphingosylphosphorylcholine (SPC) and Lysophosphatidylcholine (LPC) Cause Ca^{2+} Signaling in Enteric Glia.

Previously, to determine whether cultured guinea pig enteric glial cells were responsive to bioactive lipids, mixed primary cultures of myenteric plexus were exposed to an array of related lipid compounds. Those studies revealed that only enteric glia selectively underwent Ca^{2+} signaling in response to a variety of, but not all, lipids including sphingosine-1-phosphate (S1P), LPA (1μM) and SPC (1μM). Interestingly, enteric neurons did not display Ca^{2+} signaling following treatment with S1P, LPA, SPC, or any other lipids evualated. Similarly for these studies, no neurons responded to LPC.

Perfusion of glial cells with SPC (10 μM) and LPC (10 μM) (doses utilized routinely in the literature) for 120s caused strong Ca^{2+} mobilization in (100%, $\Delta [Ca^{2+}]_i$ = 313±27 nM, n=112) and (100%, $\Delta [Ca^{2+}]_i$ =408±35 nM, n=109) of glial cells, respectively. Representative traces are shown in Figures 5.1 and 5.2 for SPC and LPC, respectively.

SPC- and LPC-mediated Ca^{2+} Signaling Desensitizes with Repetitive Exposure.

Repetitive exposure to either SPC or LPC produced progressive decrements in calcium transients generated in enteric glia [Figures 5.3 and 5.4]. For SPC, an approximately 76% decrease in peak $[Ca^{2+}]_i$ responses was observed between the first and second exposures, and another decrement of 14% between the second and third exposures. 100% of cells responded initially to SPC and 35% completely desensitized (n=107). For LPC, an approximately 48% decrease in Δ $[Ca^{2+}]_i$ responses was observed between the first and second exposures, and another decrement of 34% between the second and third exposures. 100% of cells responded to LPC and 13% completely desensitized (n=98). To demonstrate that the effect was not due to depletion of intracellular Ca^{2+} stores, cells were subsequently perfused with ATP (100 μM) at the end of each experiment and typical increments in $[Ca^{2+}]_i$ were achieved. Desensitization is typical of receptor-mediated events.

SPC and LPC Responses are Differentially Sensitive to Pertussis Toxin (PTX).

To investigate the involvement of a PTX-sensitive G protein, enteric glia were preincubated with PTX at 100 ng/ml for 24 h, a concentration and preincubation time found to be effective in earlier studies. The Δ $[Ca^{2+}]_i$ generated by SPC in all glia pre-exposed to PTX was 62 ± 34 nM (n=97), relative to the peak $[Ca^{2+}]_i$ evoked in control glia of 243 ± 29 nM (n=131). An averaged tracing from an individual run (n=36) is included (Figure 5.5). The percentage of cells responding to SPC was also different in these two

groups: 41% vs. 100% for PTX-treated and non PTX-treated cells, respectively. The Δ $[Ca^{2+}]_i$ generated by LPC in glia pre-exposed to PTX was 481±37 nM (n=116), relative to the Δ $[Ca^{2+}]_i$ evoked in control glia of 409±28 nM (n=140). An averaged tracing from an individual run (n=56) is included [Figure 5.6]. The percentage of cells responding to LPC was identical in the two groups: 100% and 100% for PTX-treated and non PTX-treated cells, respectively.

Discussion

SPC and LPC represent two additional lysolipids in the complex and developing picture of bioactive lipid signaling in enteric glia. This report addresses in brief fashion their effects in these cells and presents evidence which supports a role for receptor-mediated SPC and LPC calcium signaling. In enteric glia, SPC and LPC cause robust calcium signaling which is rapidly desensitized and differentially sensitive to pertuss-toxin pre-treatment.

That SPC and LPC have diverse biological effects has been known for quite some time. However, the finding that they activate cell surface receptors has added to the intrigue of these compounds. They join a list of growing bioactive lipids which retain the ability to function through G protein-coupled receptors. This dissertation has already characterized the effects of S1P and LPA in enteric glia and, to this end, the discovery of SPC and LPC actions now raise the level of diversity exhibited by these cells.

This section serves as an abbreviated account of SPC and LPC functioning via receptor-mediated means in enteroglia. Lacking are data that enteric glia express any of

the receptors discussed herein. However, while it is not shown that glial cells of the gastrointestinal tract express GPR4, G2A, or OGR1, the responsiveness of these cells is consistent with receptor-mediated events. In fact, data have already been presented to show that two SPC-activated Endothelial Differentiation Gene receptors (EDG-1 and EDG-3) are present in enteric glia (see Chapter 2). Rapid desensitization is typical of receptor-mediated events, as was observed following SPC and LPC treatment. While SPC-generated calcium signals were PTX-sensitive, those generated by LPC were not, showing divergence of signaling pathways. This finding was a bit surprising since the receptors identified to date for either SPC or LPC are all at least partially coupled to the protein G_i following receptor activation. Accordingly, it remains possible that LPC is coupled to an as of yet unidentified receptor in enteric glia. Future work will need to be directed towards both further characterizing the receptor-mediated signaling events in enteric glia in response to these molecules and elucidating their role in gastrointestinal physiology.

Figures

Figure 5.1. Sphingosylphosphorylcholine (SPC) causes Ca^{2+} signaling in enteric glia. Cultured enteric glial cells exposed to 10 μM SPC. Tracing is a representative sample of over 50 cells from a single experiment.

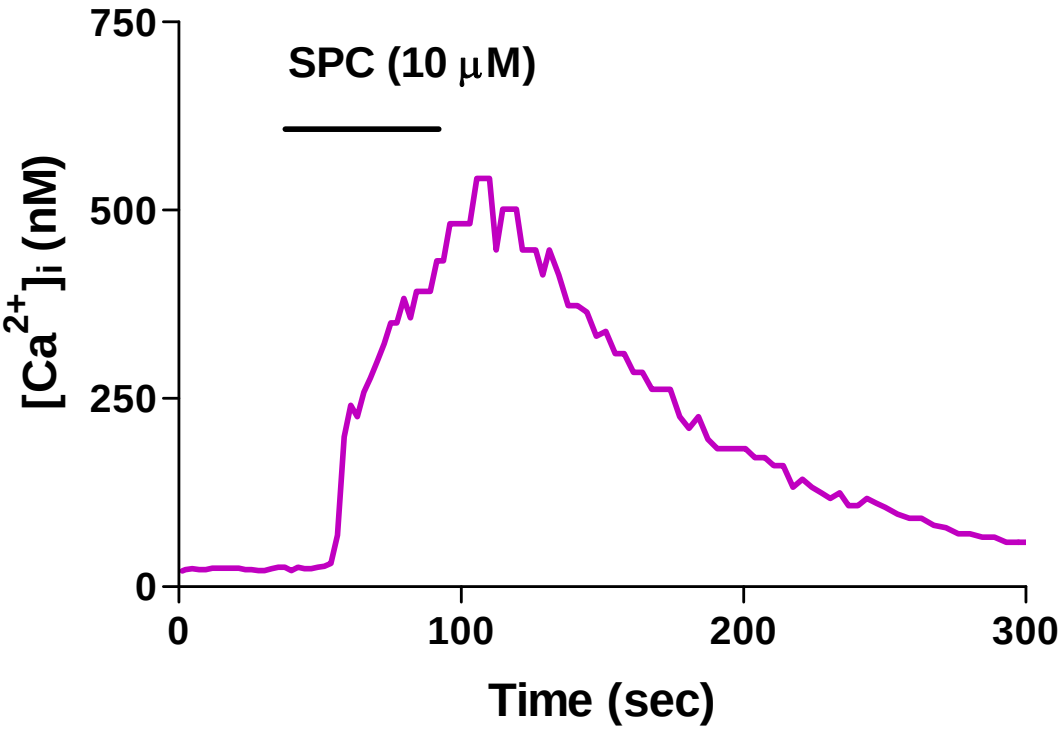


FIGURE 5.1

Figure 5.2. Lysophosphatidylcholine (LPC) causes Ca^{2+} signaling in enteric glia.

Cultured enteric glial cells exposed to 10 μM LPC. Tracing is a representative sample of over 50 cells from a single experiment.

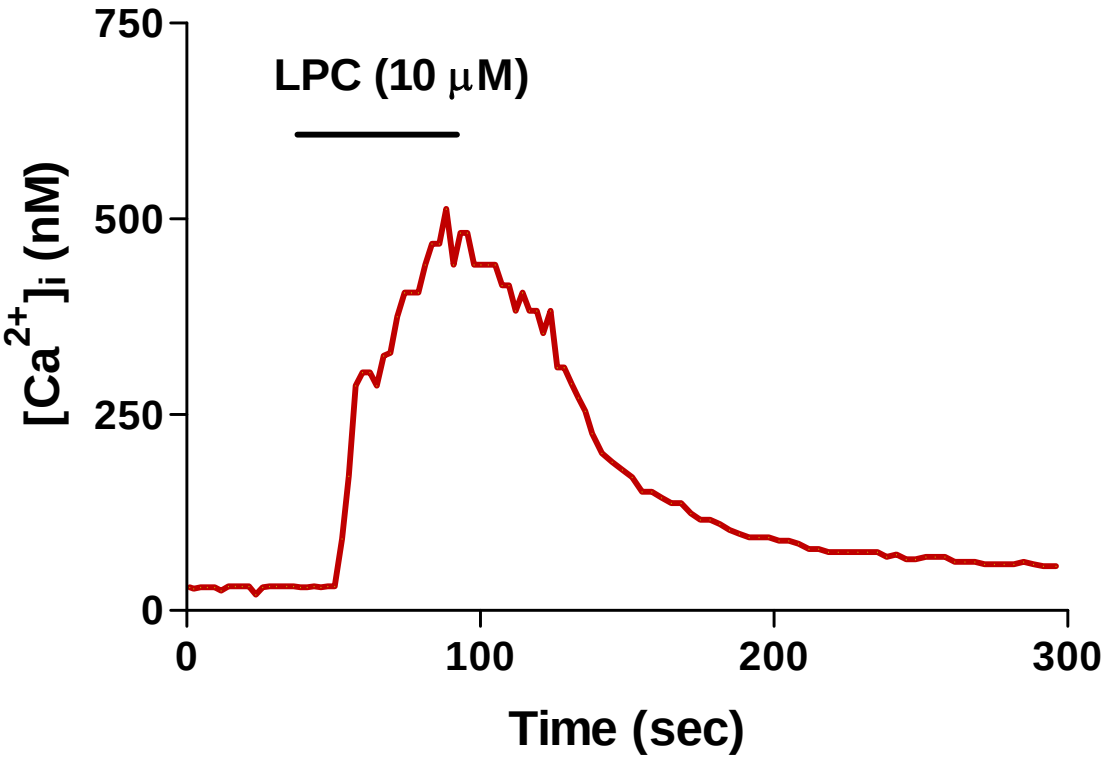


FIGURE 5.2

Figure 5.3. SPC-mediated Ca^{2+} signaling desensitizes with repetitive exposure. Cultured enteric glial cells exposed to 10 μM SPC sequentially after periods of agonist washout and recovery followed by stimulation by ATP (100 μM) to demonstrate intracellular calcium stores are not depleted. Tracing is a representative sample of over 50 cells from a single experiment.

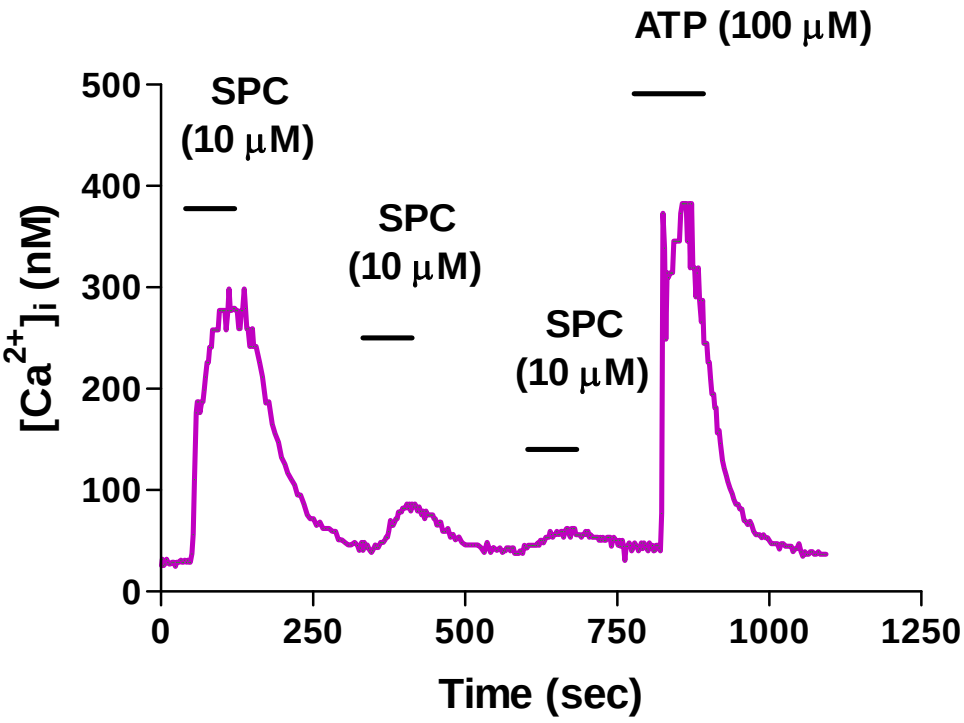

ATP (100 μM)
SPC
(10 μM)
SPC
(10 μM)
SPC
(10 μM)
500
400
300
200
100
0
[Ca2+]i (nM)
0
250
500
750
1000
1250
Time (sec)

FIGURE 5.3

Figure 5.4. LPC-mediated Ca^{2+} signaling desensitizes with repetitive exposure. Cultured enteric glial cells exposed to 10 μM LPC sequentially after periods of agonist washout and recovery followed by stimulation by ATP (100 μM) to demonstrate intracellular calcium stores are not depleted. Tracing is a representative sample of over 50 cells from a single experiment.

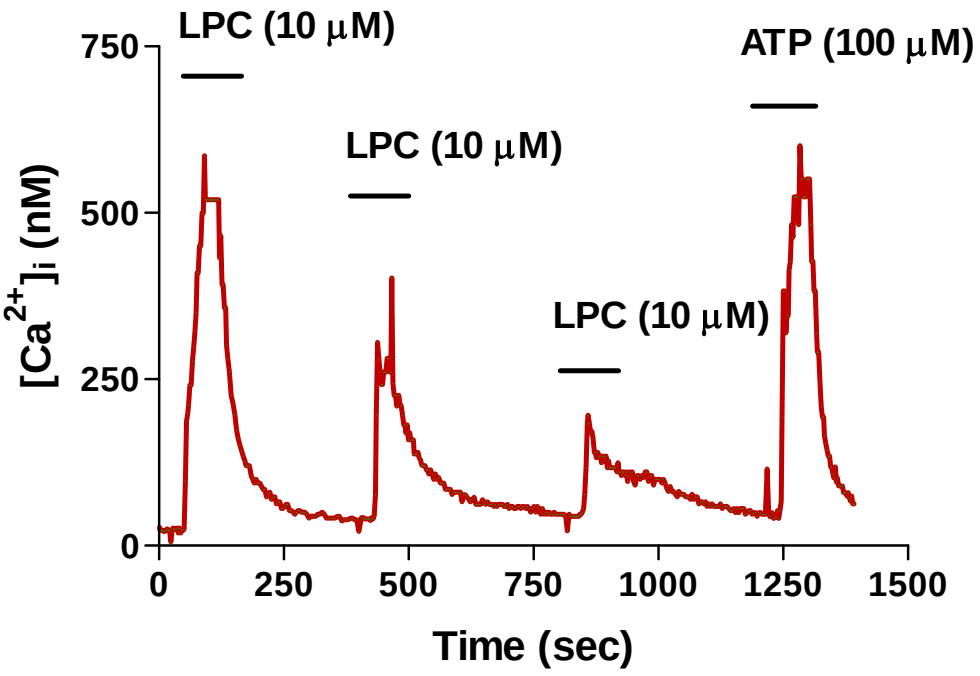

LPC (10 μM)
LPC (10 μM)
LPC (10 μM)
ATP (100 μM)
750
500
250
0
[Ca2+]i (nM)
0
250
500
750
1000
1250
1500
Time (sec)

FIGURE 5.4

Figure 5.5. SPC responses are sensitive to pertussis toxin (PTX). Cultured enteric glial cells were incubated with 100 ng/ml PTX for 24 h and then exposed to 10 μM SPC. Following pretreatment, cells display diminished responsiveness to SPC as shown. Tracing represents a compilation of over 50 cells from a single experiment.

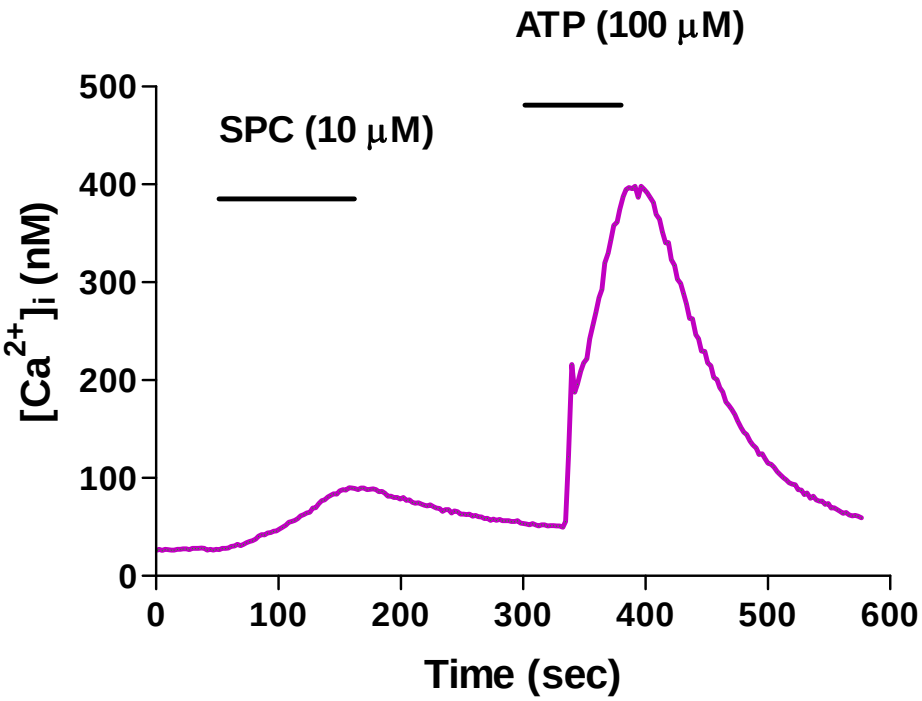

ATP (100 μM)
SPC (10 μM)
500
400
300
200
100
0
[Ca2+]i (nM)
0
100
200
300
400
500
600
Time (sec)

FIGURE 5.5

Figure 5.6. LPC responses are not sensitive to pertussis toxin (PTX). Cultured enteric glial cells were incubated with 100 ng/ml PTX for 24 h and then exposed to 10 μM LPC. Following pretreatment, cells remain responsive to LPC as shown. Tracing represents a compilation of over 50 cells from a single experiment.

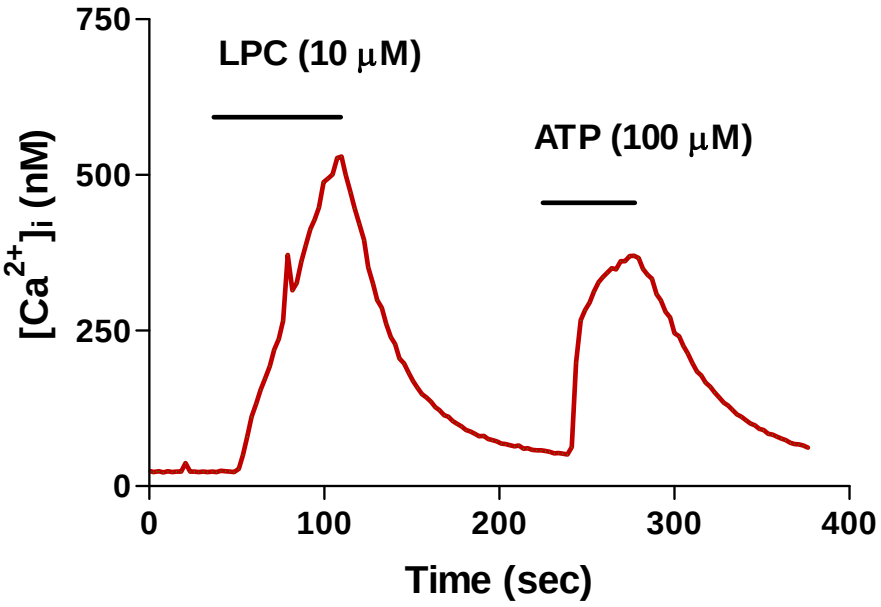
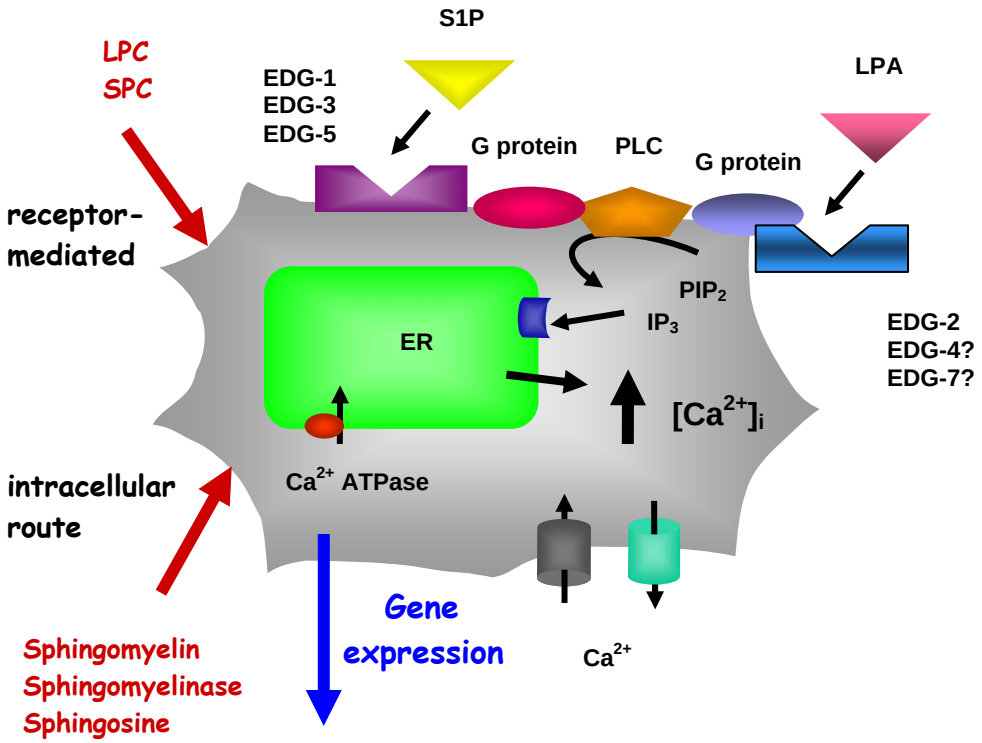

750
500
250
0
LPC (10 μM)
ATP (100 μM)
[Ca2+]i (nM)
0
100
200
300
400
Time (sec)

FIGURE 5.6

Figure 5.7. Summary of bioactive lipid signaling in enteric glia. See text for details.



?

ENS plasticity
Information transfer
Inflammation
Cytoloskeleton changes

FIGURE 5.7

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